THE ROLE OF DNA METHYLATION IN REGULATING *LHX3* GENE EXPRESSION

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Abstract

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LIM homeodomain 3 (LHX3) is an important regulator of pituitary and nervous system development. To date, twelve *LHX3* gene mutations have been identified in patients with combined pituitary hormone deficiency disease (CPHD). Understanding the molecular mechanisms governing *LHX3/Lhx3* gene regulation will provide critical insights into organ development pathways and associated diseases. DNA methylation has been implicated in gene regulation in multiple physiological systems. This dissertation examines the role of DNA methylation in regulating the murine *Lhx3* gene. To determine if demethylation of the *Lhx3* gene promoter would induce its expression, murine pre-somatotrope pituitary cells that do not normally express *Lhx3 (*Pit-1/0 cells) were treated with the demethylating reagent, 5-Aza-2'-deoxycytidine. This treatment lead to activation of the *Lhx3* gene and thus suggested that methylation contributes to *Lhx3* gene regulation. Proteins that modify chromatin, such as histone deacetylases (HDACs) have also been shown to affect DNA methylation patterns and subsequent gene activation. Pit-1/0 pituitary cells treated with a combination of the demethylating reagent and the HDAC inhibitor, Trichostatin A led to activation of the *Lhx3* gene, suggesting crosstalk between DNA methylation and histone modification processes. To assess DNA methylation levels, treated and untreated Pit-1/0 genomic DNA were subjected to bisulfite conversion and sequencing. Treated Pit-1/0 cells had decreased methylation compared to untreated

cells. Chromatin immunoprecipitation assays demonstrated interactions between the methyl-binding protein, MeCP2 and the *Lhx3* promoter regions in the Pit-1/0 cell line. Overall, the study demonstrates that DNA methylation patterns of the *Lhx3* gene are associated with its expression status.

Simon J. Rhodes, Ph.D.– Chair

Table of Contents

List of Figures..... viii

Abbreviations ix

Introduction.....1

1. The Pituitary Gland.....1

1.1 Pituitary organogenesis 2

1.2 Pituitary transcription factors 2

1.2.1 PIT-1.....2

1.2.2 LHX3.....5

2. LHX3 and Combined Pituitary Hormone Deficiency Disease 8

2.1 LHX3 T194R.....9

2.2 LHX3 W224Ter 10

3. Epigenetics.....11

3.1 Chromatin structure.....11

3.2 Histone modifications.....12

3.2.1 Interactions of the LHX3 C-terminus with the chromatin regulating complex, Inhibitor of Histone Acetyltransferase (INHAT) 16

3.3 DNA methylation 18

3.3.1 Methyl CpG binding proteins.....19

3.4 MeCP2 and chromatin crosstalk.....21

4. Focus of Dissertation 23

Methods.....31

1. Bioinformatic Analysis 31

2. Cell Culture 31

3. Cell Culture Treatments 31

4. Bisulfite Sequencing 32

5. RNA Isolation and Reverse Transcription 33

6. Protein Analysis 34

6.1 Protein isolation 34

6.2 Western blot 34

7. Chromatin Immunoprecipitation Assay 35

Results 38

1. Analysis of *Lhx3* mRNA and LHX3 Protein Expression 38

2. Identification and Investigation of Methylated Regions in *Lhx3* Promoters 38

3. 5-Aza-2'-deoxycitidine Induces *Lhx3* Gene Expression 39

4. Trichostatin A and 5-aza-dc Treatment Induce *Lhx3* Expression 40

5. Inhibitor treatment did not induce *αGSU or TSHβ* gene expression 40

6. The Methyl-Binding Protein, MeCP2 Occupies *Lhx3* Promoters 41

Discussion 52

References 62

Curriculum Vitae

List of Figures

Figure 1. Transcription cascade governing anterior pituitary development.....25

Figure 2. The *LHX3* gene 26

Figure 3. *LHX3* functional domains 27

Figure 4. Biochemical analysis of T194R mutant.....28

Figure 5. *Lhx3*$^{W227ter/W227ter}$ mice are dwarfed and have a PRL deficiency 29

Figure 6. Characterization of the LHX3-INHAT relationship 30

Figure 7. *Lhx3* mRNA and LHX3 protein expression in Pit-1/Triple and Pit-1/0 cells 43

Figure 8. *Lhx3a* promoter and its methylation pattern 44

Figure 9. *Lhx3b* and its methylation pattern.....45

Figure 10. *Lhx3* gene expression changes after treatment with 5-aza-dc in Pit-1/0 cells 46

Figure 11. Bisulfite sequencing of the *Lhx3* promoter post 5-aza-treatment 47

Figure 12. *Lhx3* gene expression level varies with concentration of 5-aza-dc plus TSA.....48

Figure 13. *Lhx3* gene expression is activated after treatment with 5-aza-dc plus TSA in Pit-1/0 cells 49

Figure 14. *αGSU* and *TSHβ* transcripts are not expressed in treated Pit-1/0 cells 50

Figure 15. MeCP2 binds the *Lhx3* gene promoters in Pit-1/0 cells.....51

Abbreviations

5-aza-dc	5-Aza-2’-deoxycytidine
ACTH	adrenocorticotrophic hormone
BAC	bacterial artificial chromosome
BDNF	brain-derived neurotropic factor
BMP	bone morphogenetic protein
BTB/POZ	broad complex, tramtrak, bric à brac/pox virus and zinc finger domain
CBP	3’-5’-cyclic adenosine monophosphate response binding element -binding protein
CpG	cytosine-phosphate bond-guanine
CPHD	combined pituitary hormone deficiency disease
CRE	3’-5’-cyclic adenosine monophosphate response element
DNMT	deoxyribonucleic acid methyltransferases
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
GH	growth hormone
Glut 3	glucose transporter 3
GNAT	Gcn-5-related N-acetyltransferase
GnRH-R	gonadotropin-releasing hormone receptor
GR	glucocorticoid receptor
GST	glutathione S-transferase
HAT	histone acetyltransferases
HDAC	histone deacetlyase
INHAT	inhibitor of histone acetyltransferase

ISL1	islet 1
LANP	leucine-rich acidic nuclear protein
LH	luteinizing hormone
LHX3	LIM homoeodomain 3
LHX4	LIM homoedomain 4
LIM	Lin11,Isl-1, Mec-3
MBD	methyl-CpG binding domain protein
MBP	methyl CpG binding protein
MYST	MOZ, Ybf2-sas3, Sas3 and Tip60
NaCh II	type II sodium channel
NF1	nuclear factor 1
PCAF	CREB-binding protein associate factor
PGBE	pituitary glycoprotein binding element
PIT-1	pituitary-specific transcriptional factor-1
PITX1	pituitary homeobox 1
POMC	proopiomelanocortin
PRL	prolactin
PRMT	protein arginine methyltransferases
PROP-1	prophet of Pit-1
SET	Su(var) 3-9,Enhancer of zeste [e(z)], and Trithorax
SP1	specificity protein 1
SUMO	small ubiquitin modifier
TAF-1β	template activating factor

Tag	T-antigen oncoprotein
TRH	thyrotropin- releasing hormone
TSA	trichostatin A
TSH	thyroid-stimulating hormone
αGSU	alpha-glycoprotein subunit

INTRODUCTION

1. The Anterior Pituitary Gland

The anterior pituitary gland regulates biological processes such as metabolism, growth, lactation, reproduction and stress. It controls these physiological and developmental events by producing hormones from five different hormone-secreting cell types—corticotropes, gonadotropes, thyrotropes, somatotropes and lactotropes. The corticotropes produce adrenocorticotropic hormone (ACTH) to regulate the stress response. ACTH is formed from the proteolytic cleavage of a precursor protein, proopiomelanocortin (POMC) (Stevens and White, 2010). Gonadotropes produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to regulate reproduction, while thyrotropes produce thyroid-stimulating hormone (TSH) to regulate metabolism. FSH, LH and TSH are heterodimeric proteins composed of a common subunit, alpha-glycoprotein (αGSU) and a specific beta subunit (FSHβ, LHβ and TSHβ) (reviewed by (Savage et al., 2003; Zhu et al., 2007)). Somatotropes produce growth hormone (GH) to regulate growth. In humans, there is a cluster of five GH-related genes in which the most 5′ gene (*hGH-N* or *GH1*) is transcribed exclusively in somatotropes and lactosomatotropes of the anterior pituitary, which produce the pituitary GH protein (Savage et al., 2003; Baumann, 2009). Lactotropes produce prolactin (PRL) to regulate lactation and the human *prolactin* locus consists of a single gene driven by two promoters—a pituitary-specific promoter and an alternative promoter which drives expression in non-pituitary tissues (Featherstone et al., 2012).

1.1 Pituitary organogenesis

The pituitary relays signals between the hypothalamus and peripheral target organs (Zhu et al., 2007). The pituitary, located in the sella turcica of the sphenoid bone at the base of the brain, is composed of two distinct parts that are derived from two separate embryological sources (Savage et al., 2003; Kelberman et al., 2009). The adenohypophysis, comprising the anterior and intermediate lobes, arises from the oral ectoderm-derived Rathke's pouch, while the neurohypophysis, also known as the posterior lobe, arises from the neural-derived infundibulum of the diencephalon. Inductive signaling between the diencephalon and Rathke's pouch initiates a transcription factor cascade leading to the development of a mature anterior pituitary, which contains the five distinct hormone-secreting cell types (Figure 1). In this process, the diencephalon signaling factors, including the key factors bone morphogenetic protein (BMP) 4 and fibroblast growth factor (FGF) 8 induce the expression of the LIN11, ISL1, MEC3 (LIM)-homeodomain 3 (LHX3) and the LIM-homeodomain 4 (LHX4) transcription factors that are essential for the development of a rudimentary Rathke's pouch and subsequent pituitary development (Hunter and Rhodes, 2005; Alatzoglou et al., 2009).

1.2 Pituitary transcription factors

1.2.1 PIT-1

Pituitary-specific transcription factor-1 (PIT-1, also known as POU1F1) plays a critical role in somatotrope, lactotrope and thyrotrope cell development. It regulates genes such as *Gh, Prl, Tshβ, growth hormone-releasing hormone* (*Ghrh) receptor* and *thyroid hormone receptor beta type 2* and its own gene through autoregulation (Andersen and Rosenfeld, 1994; Rhodes et al., 1996). The vital role of the PIT-1 in pituitary

development is exemplified in the Snell (*dw)* and Jackson (*dw^{j})* mouse models, which show thyrotrope, somatotrope and lactotrope cell deficiencies (Li et al., 1990). The Snell mouse *dw* phenotype occurs as a result of a *Pit-1* gene mutation (Li et al., 1990; Fang et al., 2011). The mutation alters a conserved amino acid in the DNA-binding POU homeodomain of the transcription factor, ultimately preventing PIT-1 from binding key regulatory regions of anterior pituitary target genes (Li et al., 1990). The other mouse model, known as the Jackson dwarf mouse, carries an inactivating *Pit-1* gene rearrangement (Li et al., 1990). The resulting *dw^{j}* phenotype is similar to the Snell phenotype showing deficiencies in GH, TSH and PRL and to human diseases featuring *PIT-1* gene mutations, suggesting that PIT-1 is a critical regulator of human pituitary gene functions (Li et al., 1990; Pfaffle and Klammt, 2011).

The regulation of the Pit-1gene and PIT-1 structure

The *Pit-1/POU1F1* gene is well characterized in rodents and humans and its gene expression is restricted to the caudomedial region of the pituitary gland (Zhu et al., 2007). In rodents, the *Pit-1* proximal promoter is a 300 bp region surrounding the transcription start site. It contains a TATA box, two cyclic AMP response elements (CREs) in the rat or one CRE in the mouse and two autoregulatory PIT-1 binding sites. The positive PIT-1 autoregulatory site is upstream, while a negative site is downstream and position dependent (McCormick et al., 1990; de la Hoya et al., 1998). The rodent *Pit-1* gene also has a distal enhancer containing additional PIT-1 autoregulatory binding sites, a vitamin D receptor element, a PIT-1 retinoic acid response element and a putative Prophet of Pit-1 (PROP-1) binding site (Rhodes et al., 1993; DiMattia et al., 1997; de la Hoya et al., 1998).

The human *PIT-1* minimal promoter spans nucleotides -102 to +15 relative to the Pit-1 transcription start site, and contains an autoregulatory PIT-1 binding element as well as additional *cis* elements that confer high basal transcriptional activity (Delhase et al., 1996). Unlike the rodent promoter sequence, the human proximal and distal promoters do not contain a CRE (de la Hoya et al., 1998). Additionally, the distal promoter region contains autoregulatory binding sites, but does not show interaction with the minimal promoter (Delhase et al., 1996). Expression of the human *PIT-1* gene is also down regulated by the OCT-1 and AP-1 transcription factors (Delhase et al., 1996).

PIT-1 belongs to the POU homeodomain family. The protein is comprised of an amino (N-) terminal activation domain and two domains responsible for high affinity DNA binding: a POU-specific domain that is located closer to the N-terminus and a carboxyl (C-)terminal POU homeodomain. The PIT-1 protein recognizes AT-rich nucleotide sequences in the pituitary-expressed *GH*, *PRL* and *TSHβ* promoters (Kerr et al., 2008)

Pit-1 lineage cell lines

Recently, Sizova and colleagues characterized three new mouse cell lines representing PIT-1-associated cell lineages (Sizova et al., 2010). Mouse *Pit-1* or *GH* gene regulatory sequences (*Pit-Tag* or *GH-Tag*) were used to guide expression of the SV40 large T-antigen oncoprotein (Tag) to the developing pituitaries of transgenic mice. Three cell lines were derived from tumors: Pit-1/0, Pit-1/Triple and Pit-1/Prl and each cell line represents different phases of PIT-1-dependent cell differentiation in the mouse anterior pituitary. The Pit-1/0 cell line, established from a pituitary tumor of a 12 week mouse carrying the *Pit-Tag* transgene and the *hGH/bacterial artificial chromosome* (*BAC)*

transgene, represents an initial stage of somato-lacto-thyrotropic differentiation. The *Pit-1* gene is expressed, but the cells fail to express the PIT-1-dependent hormones: GH, PRL and TSHβ. The Pit-1/Triple cell line, established from a 20 week mouse carrying a Pit-Tag induced pituitary tumor, represents a more differentiated Pit-1 lineage cell line. These cells express PIT-1 protein and its target genes, *Gh, Prl* and *Tshβ.* The third cell line, the Pit-1/Prl cells were harvested from the pituitary tumor of a mouse carrying both the *GH-Tag* and *hGH/BAC* transgene. The cells express PIT-1 and Prl, but not GH or TSHβ. These PIT-1 lineage cell lines are valuable tools to understand the intermediate stages of anterior pituitary development, including epigenetic changes that dictate pituitary gene expression.

1.2.2 LHX3

LHX3 (also known as LIM3 or P-Lim) plays a key role in anterior pituitary organogenesis, as well as nervous system development (Seidah et al., 1994; Bach et al., 1995; Zhadanov et al., 1995). In the pituitary, LHX3 is required for the formation of somatotropes, thyrotropes, gonadotropes and lactotropes, four of the five distinct hormone-secreting cell types. LHX3 activates the transcription of hormone genes, such as the *αGSU, PRL, FSHβ* and *gonadotropin-releasing hormone receptor (GnRH-R),* as well as the *PIT-1* transcription factor genes (Savage et al., 2003; Zhu et al., 2007). LHX3 binds the pituitary glycoprotein binding element (PGBE) of the *αGSU* gene to activate its transcription. In the nervous system, LHX3 interacts with key proteins to establish motor neuron and interneuron identity. Interaction between LHX3 and the NLI LIM cofactor is associated with V2 interneuron differentiation, while motor neuron development requires LHX3 interaction with Islet 1 (ISL1) (Thaler et al., 2002). The critical role that LHX3

plays in nervous system and pituitary development is demonstrated by the phenotype of *Lhx3* gene knockout mice (*Lhx3*$^{-/-}$) which die at birth (Sheng et al., 1996). The *Lhx3*$^{-/-}$ mice lack correctly formed anterior and intermediate pituitary lobes, and four of the five differentiated hormone-secreting anterior cell types are absent, with only a small population of corticotropes remaining (Sheng et al., 1996). Homozygous *Lhx3* knockout mice also do not undergo proper motor neuron specification (Sheng et al., 1996; Sharma et al., 1998).

The regulation of the Lhx3 gene and LHX3 structure

The *Lhx3* gene, comprising seven exons and six introns (Figure 2), produces two mRNA transcripts: *Lhx3a* and *Lhx3b.* The *Lhx3* gene contains two TATA-less, GC rich promoters that initiate the transcription of *Lhx3a* and *Lhx3b* mRNAs. Basal gene activity is mediated by the 2 kb and 1.8 kb *Lhx3a* and *Lhx3b* promoters, respectively (Yaden et al., 2006). The LHX3 promoters contain both nuclear factor 1 (NF1) and specificity protein 1 (SP1) transcription factor binding sites and both factors regulate LHX3 transcription (Yaden et al., 2006). Recently, it has also been shown that SOX2, a developmental transcription factor, can bind and activate the human *LHX3a* promoter (Rajab et al., 2008; Alatzoglou et al., 2009).

In the human *LHX3* gene, a distal enhancer confers some aspects of spatial and temporal *LHX3* expression in the pituitary and nervous system. A defined 180 bp core region (Core R3) of this enhancer guides pituitary-specific expression in model transgenes (Mullen et al., 2012). Pituitary Core R3 enhancer activity involves the binding of the ISL1 LIM-homeodomain transcription factor, and the pituitary homeobox 1

(PITX1) transcription factor, as demonstrated by chromatin immunoprecipitation assays and mutation of transgenes (Mullen et al., 2012).

The *LHX3* transcripts are translated to two major protein isoforms, LHX3a and LHX3b (Sloop et al., 1999). Each LHX3 isoform possesses distinct DNA binding and promoter activation activities (Sloop et al., 1999; Sloop et al., 2001). The LHX3a and LHX3b isoforms result from translation of the first methionine codon of the respective mRNA (Sloop et al., 1999). The LHX3 protein isoforms have specific domains that exert their functions (Figure 3) (Sloop et al., 1999; Parker et al., 2000; Sloop et al., 2001; Parker et al., 2005). Both the LHX3 protein isoforms contain a DNA-binding homeodomain, two LIM domains, which participate in protein-protein interactions, and a carboxyl terminus (C-terminus) *trans*-activation domain. The DNA-binding homeodomain also contains nuclear localization signals that target LHX3 to the nuclear matrix (Sloop et al., 1999; Parker et al., 2000). The C-terminus of the LHX3 isoforms is divided into three regions, C1, C2 and C3 (Figure 3). The C1 domain contains a nuclear localization signal and multiple phosphorylation sites (Parker et al., 2005). LHX3 is a predicted substrate of protein kinase C and casein kinase II, and overexpression of these kinases leads to a decrease in activation of LHX3 target genes. The C2 region contains a *trans*-activation domain site important for synergy with PIT-1 to activate target promoters, such as those of the prolactin and *Tshβ* genes (Bach et al., 1995; Sloop et al., 1999; Sloop et al., 2001). The function of the C3 domain has yet to be elucidated.
The LHX3a isoform acts as a transcriptional activator, while the LHX3b isoform is a poor activator of gene transcription and may serve as a repressor (Sloop et al., 1999).

A third protein isoform, M2-LHX3, can be formed by preferential translation of the second in-frame methionine codon of the *LHX3a* mRNA, and is primarily made up of the homeodomain plus the C-terminus activation domain (Sloop et al., 2001). Like LHX3a, M2-LHX3 can induce transcription of the *PRL, αGSU, FSHβ and TSHβ* genes (Sloop et al., 2001; West et al., 2004).

2. LHX3 and Combined Pituitary Hormone Deficiency Disease

Mutations and gene deletions in human pituitary transcription factor genes have been implicated in the etiology of hormone deficiency diseases. Genetic changes affecting anterior pituitary transcription factors often lead to negative downstream effects, including loss of multiple hormones (Pfaffle and Klammt, 2011). Combined pituitary hormone deficiency (CPHD) disease is diagnosed by the insufficiency of GH and one or more anterior pituitary hormones (Pfaffle and Klammt, 2011). CPHD patients are usually diagnosed as children due to the developmental nature of the affected transcription factor(s). Although rare, recessive mutations in *LHX3* are responsible for some CPHD diagnoses. To date, twelve recessive *LHX3* mutations have been molecularly and clinically identified as associated with CPHD (Netchine et al., 2000; Bhangoo et al., 2006; Pfaeffle et al., 2007; Rajab et al., 2008; Kristrom et al., 2009; Bonfig et al., 2011; Colvin et al., 2011; Bechtold-Dalla Pozza et al., 2012). Patients with recessive *LHX3* mutations have deficiencies in GH, PRL, TSH, FSH and LH. As a result of the hormone loss, patients may exhibit shortness of stature, metabolic defects and delayed/failed puberty. Other symptoms may include a hypoplastic, normal or enlarged anterior pituitary structure, rigid spine, hearing loss, and ACTH deficiency. Two interesting *LHX3* mutations, T194R and W224Ter, provide insight into the DNA-binding properties and

functional domains of the protein, respectively (Pfaeffle et al., 2007; Bechtold-Dalla Pozza et al., 2012). Understanding the molecular mechanisms associated with CPHD may provide pathways to potential therapies and treatments for patients, while enhancing our knowledge of pituitary function.

2.1 LHX3 T194R

Two sibling pediatric patients with CPHD presented with neonatal complications and one patient died 20 days after birth due to cardiorespiratory insufficiency. The surviving patient presented with sensorineural hearing defect (nervous system deficiency), as well as loss of GH, LH, FSH, TSH and PRL, with a later onset of ACTH deficiency (pituitary defect). The patients had a homozygous mutation in *LHX3* that resulted in an amino acid substitution (Bechtold-Dalla Pozza et al., 2012). For each patient, sequencing of the *LHX3* gene identified a homozygous C→G transversion in the fourth coding exon. The transversion changed amino acid 194 from threonine to arginine (T194R) in the DNA-binding homeodomain. Sequence analyses demonstrated that the threonine residue was conserved among the LHX3/ LIMs proteins of various species, as well as in the corresponding position in other human LIM-homeodomain proteins. Computer modeling predicated that the T194R substitution would lead to steric hindrances, mainly with glutamine 170 and leucine 172, suggesting that the steric hindrances may destabilize the tertiary structure of the DNA-binding homeodomain. Electrophoretic mobility shift assays (EMSA) showed that LHX3 T194 fails to bind known LHX3 target sequences, and the LHX3 T194R did not activate *luciferase* reporter genes under the control of the *αGSU* and *Prl* gene promoters (Figure 4).

The T194R mutation is of particular interest because it represents the major class of *LHX3* mutations in that it affects both pituitary and nervous system development. However, one identified *LHX3* mutation, W224Ter, is described to only affect pituitary development (Pfaeffle et al., 2007).

2.2 LHX3 W224Ter

The *LHX3* W224Ter mutation was identified in four siblings of a consanguineous Lebanese family (Pfaeffle et al., 2007). The patients presented with pituitary hormone deficiency but without the typical cervical spine rigidity that is thought to result from loss of LHX3 action in the nervous system. This phenotype therefore is unlike other *LHX3* mutation patients who have both pituitary and nervous system defects. This *LHX3* mutation was result of a G→A transition in position 672 of the *LHX3a* open reading frame, introducing a premature stop codon predicted to cause loss the of the C-terminus of the LHX3 protein (Pfaeffle et al., 2007). As described above, the LHX3 C-terminus contains *trans*-activation domains that are critical for pituitary gene regulation (Bach et al., 1995; Parker et al., 2000; Sloop et al., 2001) so it is consistent that loss of this protein region would lead to pituitary defects. A mouse model of the human *LHX3* W224Ter disease (*Lhx3* W227Ter) was created to determine the molecular and cellular outcomes from this mutation (Colvin et al., 2011). Similar to the patients, the mice displayed dwarfism, reduced body weight and hypothyroidism (Figure 5). Although patients with the W224Ter mutation had reduced FSH, LH and PRL, their inherent reproductive potential was unknown due to the necessity of hormone therapy at an early age. The female W227Ter mice exhibited infertility, with marked deficiencies in LH, FSH and PRL (Figure 5). The phenotype of the *Lhx3* W227Ter mouse model demonstrates that the

molecular actions of LHX3 in the pituitary and nervous system are separable *in vivo*, as well as confirming the role of the LHX3 C-terminus in pituitary development.

To further understand the critical role of the LHX3 C-terminus in pituitary gene transcription, our lab performed an affinity purification screen to identify proteins that interact with the C-terminus. Results from the screen indicated that chromatin-regulating proteins interact with the LHX3 C-terminus. However, a general understanding of epigenetics is necessary to further define both the interaction of the LHX3 C-terminus with the chromatin regulators, as well as regulation of the *Lhx3* gene.

3. Epigenetics

Epigenetics can be defined in various ways, depending on the context. Here, it is defined as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird, 2007). This definition, presented by British geneticist, Dr. Adrian Bird in the introduction of the May 2007 Nature Insight Epigenetics issue, aims to unify multiple epigenetic events that occur in a cell.

3.1 Chromatin structure

Genomic DNA is packaged with histone proteins to form chromatin. In the canonical model, nucleosomes, the basic unit of chromatin are composed of ~147 bp of DNA wrapped around a histone octamer composed of two copies each of the histones H2A, H2B, H3 and H4 (Kornberg and Thomas, 1974; Luger et al., 1997). Electrostatic interactions and hydrogen bonds between the DNA and histones stabilize nucleosome structure. Additionally, histones have a flexible tail, which can interact with neighboring nucleosomes or nuclear factors (Luger and Richmond, 1998). Short DNA segments, termed linker DNA, connect neighboring nucleosomes to form the primary structure of

chromatin, often referred to as “beads-on-a-string” (Oudet et al., 1975; Thoma et al., 1979; Turner, 2005; Luger et al., 2012). Nucleosomes interact with each other to form chromatin fibers, and subsequent fiber interactions lead to highly condensed chromatin. Chromatin is organized into two types of conformations, heterochromatin and euchromatin (Bassett et al., 2009). Condensed heterochromatin is inaccessible to DNA binding factors and is usually transcriptionally silent, whereas decondensed euchromatin is generally accessible and transcriptionally active (reviewed by (Grewal and Moazed, 2003)). Nucleosome modifications, resulting from post-translational modifications of histones and DNA methylation, provide a mechanism for the regulation of nuclear processes by providing an explanation of how transcription factors access DNA within the context of chromatin.

3.2 Histone modifications

Histones are subject to post-translational modifications, chemical modifications of amino acid side chains (Berger, 2002). Although there are numerous post-translational modifications, histone tails are most commonly modified by phosphorylation, SUMOylation, methylation, and acetylation.

Histone phosphorylation is linked to multiple cellular processes, including, but not limited to chromatin condensation, transcriptional regulation, and apoptosis (Wei et al., 1998; Goto et al., 1999; Cruickshank et al., 2010; Mahajan et al., 2012; Park and Kim, 2012). Chromatin phosphorylation has been implicated in chromatin condensation during the cell cycle. For example, phosphorylation of histone H3 on Ser10 and Ser28 plays a role in mitotic and meiotic chromosome condensation (Wei et al., 1998; Goto et al., 1999). Histone phosphorylation is also linked to apoptosis as demonstrated by the

findings that kinase Cδ phosphorylation of histone H3 on Ser10 occurs when cells have been exposed to death stimuli (Park and Kim, 2012). An example of histone phosphorylation affecting transcriptional regulation is demonstrated by the recent work of Mahajan and colleagues, who showed that phosphorylation of histone H2B at T37 by the WEE1 kinase leads to the transcriptional suppression of core histone genes (Mahajan et al., 2012). The cellular functions altered by histone phosphorylation are extensive, yet the above examples provide a glimpse into the diverse complexities of histone phosphorylation (Banerjee and Chakravarti, 2011).

Another histone modification is SUMOylation, which occurs when a small ubiquitin modifier (SUMO) is post-translationally added to proteins. SUMO modification has been shown to regulate protein-protein interactions by increasing the affinity of interacting proteins, as well as altering the subcellular localization of modified proteins (Lomeli and Vazquez, 2011). The addition of the SUMO moiety has also been shown to protect proteins from ubiquitin-dependent degradation (Melchior, 2000). With regard to histones, Shiio and Eisenman showed that SUMOylation of histone H4 leads to gene silencing through the recruitment of histone deacetylase and heterochromatin protein 1 (Shiio and Eisenman, 2003).

A third type of histone modification is methylation, which occurs when histone methyltransferases target lysine or arginine residues. A family of nine protein arginine methyltransferases (PRMTs) transfer a methyl group to the arginine residue of histone proteins, while lysine residues are usually modified by the Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain family of methyltransferases (Berger, 2002). Histone methylation can either activate or repress gene transcription. For example methylation of

histone H3 at lysine 9 (H3K9) can be a marker of gene repression, while methylation of histone 3 at lysine 4 (H3K4) has been associated with activation of specific genes (Lee et al., 2005).

Acetylation is a well characterized post-translational histone modification. The addition of an acetyl group is catalyzed by histone acetyltransferases (HATs). There are three "superfamilies" of HATs: the GNAT (Gcn5-related N-acetyltransferase), the MYST (MOZ, Ybf2-sas3, Sas2 and Tip60), and the p300/CREB-binding protein (CBP) families (Verdone et al., 2006; Graff and Tsai, 2013). In mammals, the GNAT family has homologues in various organisms and is divided into two subfamilies named the GCN5 acetyltransferases and the p300/CREB-binding associated factors (PCAF) (Verdone et al., 2006). Both proteins are associated with HAT activity, but PCAF has also been shown to act as a co-activator in cellular processes, such as nuclear-receptor mediated activation and hormone promoter regulation (Blanco et al., 1998; Nagy and Tora, 2007; Wang et al., 2010; Kino and Chrousos, 2011). In the pituitary *TSHβ* promoter, cyclic adenosine monophosphate (cAMP) treatment led to increased binding of PCAF and p300 to the promoter, and subsequent increased H4 acetylation, suggesting that recruitment of the HATs likely caused the observed acetylation (Wang et al., 2010). The p300/CBP HAT subfamily also has roles in hormone and developmental transcription factor regulation (Hashimoto et al., 2000; Hashimoto et al., 2005; Miller et al., 2012). Hashimoto and colleagues demonstrated that after thyrotropin-releasing hormone (TRH) stimulation, LHX3a and CBP synergistically activated the mouse *αGSU* gene promoter (Hashimoto et al., 2005). PIT-1 has also been shown to recruit coactivator complexes containing CBP/p300 to activate gene transcription (Xu et al., 1998; Zanger et al., 1999).

Histone acetylation is important for regulation, so it follows that the modification is reversible.

Histone deacetylases (HDACs) catalyze the removal of the acetyl group from histones. Proteins with HDAC activity can be divided into three main classes based on homology with the yeast proteins: Rpd3, Hos 1 and Hos 2 (class I), HDA1 and Hos3 (class II) and the sirtuins (class III) (Gallinari et al., 2007). HDAC11, found in higher eukaryotes, is regarded as a separate class (class IV) due to its different phylogenetics (Gallinari et al., 2007). Class III HDACs depend on NAD+ as a cofactor for the transfer of the acetyl group, whereas class I, II and IV enzymes depend on a zinc-catalyzed hydrolysis of the acetyl-amide bond by a zinc (de Ruijter et al., 2003).

HDACs interact with other proteins to modulate gene expression. In the pituitary, histone H4 is deacetylated by the ligand-dependent recruitment of the glucocorticoid receptor (GR) and HDAC2 to the *POMC* locus, leading to gene repression (Bilodeau et al., 2006). HDACs have also been identified as members of corepressor complexes, including mSin3A, NURD/Mi2/NRD and CoREST corepressor complexes (Gallinari et al., 2007). Similar to nuclear receptors, the PIT-1 protein can interact with both co-repressor and co-activator complexes to modulate gene expression (Scully et al., 2000; Voss et al., 2005). PIT-1 interacts with the NCoR complex, which associates with HDAC proteins to modify chromatin structure and repress gene transcription (Xu et al., 1998; Scully et al., 2000; Voss et al., 2005). Wen and colleagues demonstrated that HDAC3 is involved in the NCoR mediated repression of PIT-1 (Wen et al., 2000). HDACs also associate with other chromatin remodeling proteins, such as the inhibitor of histone acetyltransferase (INHAT) complex (Kutney et al., 2004a).

3.2.1 Interactions of the LHX3 C-terminus with the chromatin regulating complex, inhibitor of histone acetyltransferase (INHAT)

As described earlier, the LHX3 C-terminus contains important activation and signaling domains (Figure 3). To further understand the role of the LHX3 C-terminus in pituitary gene regulation, putative proteins that interact with the LHX3 C-terminus were identified by an affinity purification approach coupled with mass spectrometry. Leucine-rich acidic nuclear protein (LANP) and template activating factor-1β (TAF-1β), members of the INHAT complex, were identified as putative proteins that interact with the C-terminus of LHX3. INHAT is a multi-subunit complex that inhibits histone acetylation and modulates chromatin structure.

INHAT is composed of two main subunits, LANP and TAF-1β. LANP, also known as pp32, PHAP-1, mapmodulin or I1PP2A, is a 28.5 kDa tumor suppressor protein (Li et al., 1996). It has a leucine-rich amino terminal and a long C-terminal acidic domain that inhibits histone acetylation (Seo et al., 2002). LANP has other postulated roles in the cell, including neuronal differentiation, RNA shuttling, microtubule-based functions, apoptosis and inhibition of protein phosphatase 2A (PP2A) (Matilla and Radrizzani, 2005; Kular et al., 2009). LANP also binds ataxin 1, a polyglutamine protein that is linked to neurodegenerative diseases (Matilla et al., 1997). The other INHAT subunit, TAF-1β is a 39 kDa histone chaperone encoded by the myeloid leukemia-associated oncogene *SET* (Nagata et al., 1995). *SET* was identified as a novel translocation in a leukemia patient “SE” (von Lindern et al., 1992). Like LANP, TAF-1β has a long acidic tail, which also participates in histone acetyltransferase inhibition (Seo et al., 2001). TAF-1β also has various other suggested functions in the cell, such as

inhibition of PP2A, apoptosis and cell cycle regulation (Li et al., 1996; Estanyol et al., 1999).

Our lab used molecular and biochemical techniques to characterize the INHAT-LHX3 interaction. Glutathione S-transferase (GST)-interaction assays demonstrated that the acidic tails of TAF-1β and LANP interact with LHX3, while chromatin immunoprecipitation assays revealed that the INHAT complex occupies the *αGSU* gene pituitary glycoprotein binding element (PGBE), a critical gene regulatory element recognized by LHX3 (Figure 6). Other studies have shown that INHAT proteins participate in gene repression (Seo et al., 2001; Kutney et al., 2004b; Telese et al., 2005; Macfarlan et al., 2006). Similarly, our data also indicate LHX3, LANP, and TAF-1β associate with the LHX3-responsive *αGSU* promoter in mouse gonadotrope cells, and LHX3-driven pituitary gene activation is inhibited by INHAT protein overexpression. However, interactions between INHAT and LHX3 may also prevent gene repression by INHAT. In this model, interactions of the LHX3 C-terminus with the acidic domains of TAF-1β and LANP disrupt the INHAT complex, leading to its disassociation from the promoter. The dissociation of INHAT would then allow for subsequent *trans*-activation of the gene target by LHX3 and possibly the global co-activator CBP, which has previously been shown to interact with LHX3 (Hashimoto et al., 2005). Thus, LHX3 may work both indirectly by disrupting core INHAT proteins in a "de-repression" function and directly by serving as a DNA-binding transcription factor on pituitary target genes. Collectively, the INHAT-LHX3 interaction suggests coordinated interplay between the cellular microenvironment and the developmental transcription factor, LHX3.

3.3 DNA methylation

DNA methylation represents another level of transcriptional control and regulation in the cell. DNA methyltransferases (DNMTs) catalyze the addition of a methyl group to the 5′ position of the cytosine pyrimidine ring of CpG (cytosine-phosphate bond-guanine) dinucleotides (Ramsahoye et al., 1996; Robertson and Jones, 2000). CpG islands are CG dense regions (~1 CpG per 10 bp) that are usually resistant to methylation and account for ~2% of CpG of the human genome (Illingworth et al., 2008; Smith and Meissner, 2013). However, the promoters of some developmental genes, such as *Hox* genes, have been shown by mapping experiments to have methylated CpG islands (Illingworth et al., 2008; Auclair and Weber, 2012).

DNA methylation has various roles in the cell, such as chromatin silencing, female X chromosome inactivation, the imprinting of parental alleles, viral gene inactivation and silencing of individual genes (Ramsahoye et al., 1996; Dulac, 2010). Some methylation patterns are cell heritable, meaning the maintenance methyltransferase DNMT1 uses the methylation pattern of the original DNA strand as a template for catalyzing a symmetric methylation pattern on a newly synthesized DNA strand (Otto and Walbot, 1990). Because of its role in maintenance methylation, DNMT1 is a critical enzyme during development. In mice, targeted mutation of the *Dnmt1* gene is embryonic lethal, with mice not surviving past mid-gestation (Li et al., 1992).

Another type of DNA methylation, appropriately known as *de novo* methylation, establishes new patterns of methylation in cells (Zhang and Ho, 2011). It is particularly important for early development and gametogenesis and involves DNMT3 methyltransferases (Jahner et al., 1982; Stewart et al., 1982; Okano et al., 1999). In mice,

the *Dnmt3* family is composed of the *Dnmt3a* and *Dnmt3b* genes. These genes are essential for development, as *Dnmt3a*$^{-/-}$ mice die four weeks after birth and *Dnmt3b*$^{-/-}$ mice die mid-gestation (Okano et al., 1999). Interestingly, targeted mutation of the murine *Dnmt2* gene does not alter *de novo* or maintenance methylation of proviral DNA, suggesting it is not involved in global *de novo* DNA methylation (Okano et al., 1998).

3.3.1 Methyl CpG binding proteins

DNA methylation, both maintenance and *de novo*, establishes another platform for cellular regulation. It can serve as a marker or signal for transcription factors, or other binding proteins, such as methyl CpG-binding proteins. Methyl-CpG binding proteins (MBPs) bind methylated DNA and recruit regulatory proteins to modulate chromatin structure and gene activity (Buck-Koehntop and Defossez, 2013). These proteins can be divided into two main families based on structural homology: methyl-CpG binding domain proteins (MBDs) and zinc-finger proteins (Bogdanovic and Veenstra, 2009; Buck-Koehntop and Defossez, 2013).

MBD proteins

The MBD family is composed of MeCP2, MBD1, MBD2, MBD3, MBD4, MBD5 and MBD6. However, MBD3, MBD5 and MBD6 do not directly bind methylated DNA and certain splice variants of MBD1 have been shown to bind unmethylated DNA as well (Fujita et al., 2000; Saito and Ishikawa, 2002; Laget et al., 2010). The MBD family is primarily associated with transcriptional repression mechanisms, but MBD1 and MBD4 have additional functions in DNA repair (Bellacosa et al., 1999; Watanabe et al., 2003). MeCP2 was the first MBD protein discovered and homology searches using the amino acid sequence of the MeCP2 MBD domain led to the discovery of MBD1, MBD2, MBD3

and MBD4 (Cross et al., 1997; Hendrich and Bird, 1998). MeCP2 is involved in both transcription repression and activation, with its activity dependent on the specific chromatin microenvironment (Klose and Bird, 2004; Bogdanovic and Veenstra, 2009). For example, MeCP2 has been shown to recruit chromatin remodeling complexes, such as Sin3a and repress transcription at the promoter of brain-derived neurotrophic factor (BDNF) (Chen et al., 2003; Martinowich et al., 2003; Klose and Bird, 2004). Mutations in MeCP2 are associated with Rett Syndrome, an X-linked neurodevelopment disorder in females (Amir et al., 1999).

The zinc finger methyl-binding proteins include Kaiso, ZBTB4 and ZBTB38, and like the MBDs are associated with transcriptional repression (Bogdanovic and Veenstra, 2009). Kaiso is a nuclear protein, first discovered to interact with p120 catenin (Daniel and Reynolds, 1999). Prokhortchouk and colleagues later identified Kaiso as a methylation-specific DNA binding protein with transcriptional repression activity (Prokhortchouk et al., 2001). The Kaiso protein belongs to the BTB/POZ (broad complex, tramtrak, bric à brac/pox virus and zinc finger) subfamily (Buck-Koehntop et al., 2012). The zinc finger domain recognizes DNA and the BTB/POZ domain mediates protein-protein interactions. Kaiso has three Cys_2His_2 zinc fingers with two recognizing methylated CpG dinucleotides and the other recognizing the consensus sequence TCCTGCNA (KBS motif) (Buck-Koehntop et al., 2012). The other two zinc finger methyl-binding proteins, ZBTB4 and ZBTB38 contain Kaiso-like zinc fingers, but unlike Kaiso they can bind single methylated CpGs (Filion et al., 2006).

3.4 MeCP2 and chromatin cross-talk

While it is necessary to understand the inherent properties of individual transcriptional regulators, it is the cross-talk between these factors that is helpful in elucidating mechanisms of transcriptional regulation. The interactions between DNA methyl-binding proteins and histone modifying proteins are essential for coordinated transcriptional regulation in cells. A general understanding of communication within the chromatin microenvironment of various cell types is imperative for identifying potential regulatory mechanisms in the pituitary, as these interactions may modulate the development, production and release of hormones. Multiple studies have associated various methyl-binding proteins with histone modifying complexes (reviewed by (Vaissiere et al., 2008; Bogdanovic and Veenstra, 2009)), but the interactions of MeCP2 with methylated DNA are the main focus of this dissertation.

The Cedar laboratory conducted one of the first studies linking chromatin structure with DNA methylation by examining protein-DNA interactions of mouse cells transfected with methylated or unmethylated DNA (Keshet et al., 1986). Using DNase I-sensitivity assays, they showed that unmethylated DNA integration formed DNase I-sensitive conformations, whereas methylated DNA integration prevented formation of the structure (Keshet et al., 1986). These findings suggested that the inhibitory effects of DNA silencing involve alterations in protein-DNA interactions, and thus implicated histones and other transcription factors in the mechanism.

Studies examining the interactions of methyl-binding proteins with chromatin modifiers, including HDACs and co-repressor complexes confirmed cross-talk between DNA methylation and chromatin in regulating gene expression. For example, studies in

Xenopus and mice have demonstrated that MeCP2 interacts with Sin3 and histone deacetylase to trigger deacetylation of histones (Jones et al., 1998; Nan et al., 1998). As mentioned earlier, one study demonstrated that MeCP2 recruited the mSin3a-HDAC1 complex to repress transcription of the *BDNF* promoter in cortical cells (Chen et al., 2003; Martinowich et al., 2003; Klose and Bird, 2004). MeCP2 can also interact with histone methyltransferases to mediate repression (Lunyak et al., 2002; Fuks et al., 2003). MeCP2 has been identified as a participant in neuronal gene repression, and the Rosenfeld lab performed a two stage chromatin immunoprecipitation assay to show that both MeCP2 and the histone H3 lysine 9 (H3K9) methyltransferase, SUV39H1 bind the neuronal *type II sodium channel (NaCh II)* promoter in Rat-1 cells (Lunyak et al., 2002). The lab also used ChIP assays to show that MeCP2, the CoREST co-repressor complex and SUV39H1 are present on the same *NaCh II* transcription units. In a separate study, Fuks and colleagues showed that MeCP2 enriched histone H3K9 methyltransferase activities in 293T cells (Fuks et al., 2003).

Although there is substantial evidence of the role of MeCP2 in gene repression, some studies suggest MeCP2 interacts with chromatin modifying proteins to activate genes (Jones et al., 1998; Nan et al., 1998; Lunyak et al., 2002; Chen et al., 2013). A recent study showed that MeCP2 recruits CREB1 to activate the murine neuronal *glucose transporter isoform 3 (glut3)* gene in murine neurons (Chen et al., 2013). Microarray analysis of hypothalamic tissue from MeCP2 null mice and mice that overexpress MeCP2 (MECP2-Tg mice) indicated that approximately 85% of genes appeared to be activated by MeCP2 (Chahrour et al., 2008). Additionally, CREB1 co-immunoprecipitated with MeCP2 from mouse brain extracts, and functional assays demonstrated that co-

transfection of CREB1 and MeCP2 enhanced *somatostatin* reporter activity in Neuro2a cells (Chahrour et al., 2008).

Other studies have implicated MeCP2 in modulating chromatin architecture (Ghosh et al., 2010; Skene et al., 2010; Baker et al., 2013). MeCP2 has been shown to bind linker DNA, induce nucleosome conformational changes (primarily extensive nucleosome packing) and form a competitive equilibrium with histone H1 for chromatin binding (Ghosh et al., 2010). In neurons, MeCP2 has been suggested to act as an alternate histone linker (Skene et al., 2010), as well.

Communication involving DNA methylation and histone complexes in the pituitary have yet to be elucidated, but data from Martinowhich and Sun indicate that MeCP2 binds the *Pomc* promoter (Tao et al., 2009). To further understand transcriptional regulation of gene expression in pituitary cells, future studies need to explore the interactions of DNA methylation and chromatin architecture in the pituitary.

4. Focus of Dissertation

LHX3 is a critical regulator of pituitary and nervous system development. The coordinated and controlled molecular mechanisms involved in its transcription regulate its expression. Interestingly, LHX3 is differentially expressed in murine pituitary cell lines representing distinct stages of pituitary differentiation. DNA methylation is often associated with differential gene expression, but the epigenetics associated with *Lhx3* gene expression have yet to be explored. Therefore, the focus of this dissertation is on the role of DNA methylation in regulating *Lhx3* gene expression in the murine pituitary Pit-1/0 precursor cell line. The demethylating reagent 5-Aza-2'-deoxycytidine (5-aza-dc) is commonly used to examine DNA methylation, since it inhibits DNMTs from methylating

DNA (reviewed by (Christman, 2002)). Gene activation resulting from treatment with 5-aza-dc and the HDAC inhibitor, trichostatin A (TSA) has also been demonstrated in multiple studies and thus provides a tool to study cross-talk between DNA methylation and histone modifiers (Cameron et al., 1999; Bovenzi and Momparler, 2001; Yu et al., 2010). This dissertation examines patterns of DNA methylation of the *Lhx3* promoters, examines the effect of 5-aza-dc and TSA on *Lhx3* gene expression, and identifies an association between the *Lhx3* promoters and MeCP2 in the mouse Pit-1/0 cell line. Understanding the epigenetics of the *Lhx3* gene is important because dysregulation or mutations of the *LHX3*/*Lhx3* gene results in syndromic pediatric diseases such as CPHD.

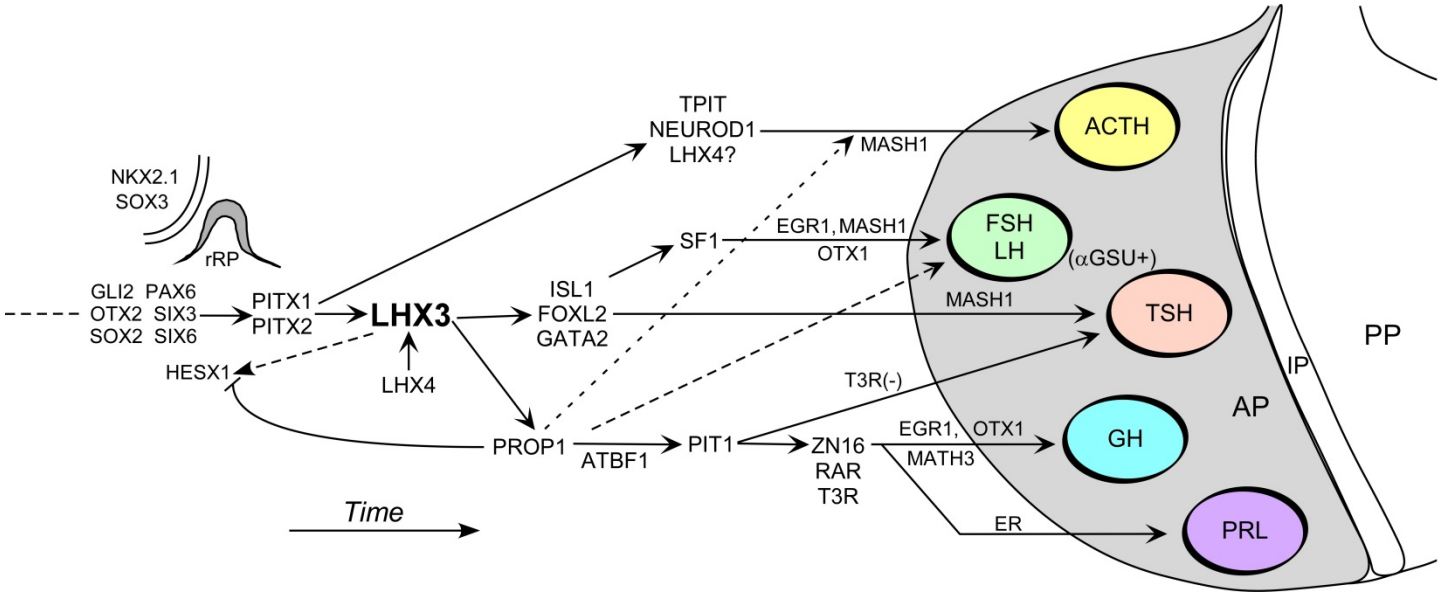

Figure 1. Transcription cascade governing anterior pituitary development (Mullen et al., 2007; Colvin et al., 2011). AP= anterior pituitary, IP = intermediate pituitary, PP= posterior pituitary, rRP= rudimentary Rathke's pouch.

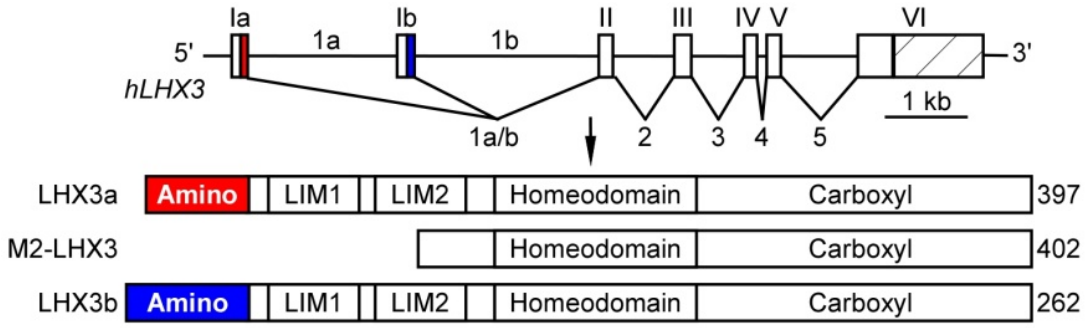

Figure 2. The *LHX3* gene. The human *LHX3* gene is located on chromosome 9 and produces two major mRNAs (*LHX3a*, *LHX3b*) encoding three protein isoforms (LHX3a, LHX3b, and M2-LHX3) with distinct activities (Sloop et al., 1999; Sloop et al., 2001).

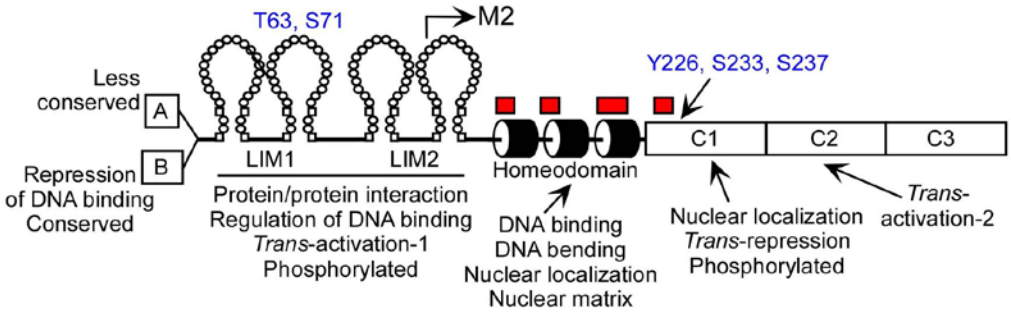

Figure 3. LHX3 "functional domains" (Sloop et al., 1999; Parker et al., 2000; Bridwell et al., 2001; Parker et al., 2005). A, B, M2: alternate amino termini. C1, C2, C3: carboxyl terminal regions. Red boxes: nuclear localization signals. Phosphorylation sites indicated in blue.

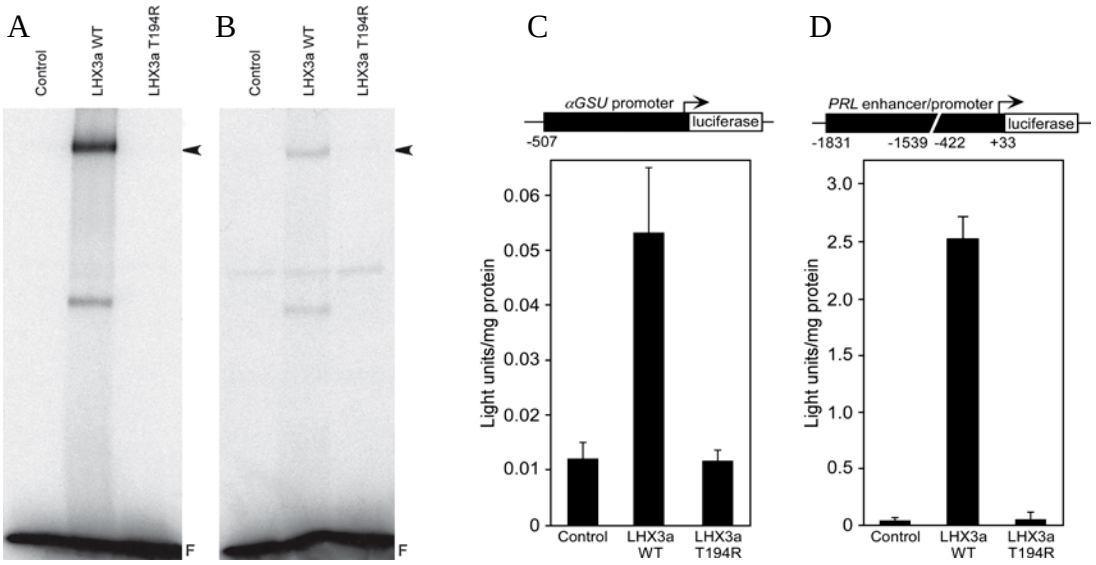

Figure 4. Biochemical analysis of the T194R "mutant" form of LHX3. (A, B) The LHX3 T194R protein does not bind to LHX3 DNA recognition elements. EMSA experiments were performed using the WT or T194R LHX3 proteins translated *in vitro* in rabbit reticulocyte lysates and radiolabeled DNA probes representing the LHX3 consensus-binding site (Bridwell et al., 2001) **(A)** or the *αGSU* promoter pituitary glycoprotein-binding element **(B)**. Unprogrammed lysates were used as negative controls (Control). The LHX3/DNA complexes are denoted by arrowheads. F = Free probe. **(C, D)** The LHX3 T194R protein is unable to activate pituitary target genes. Expression vectors for wild type (WT) and T194R LHX3 proteins were transiently cotransfected into mouse pituitary GHFT1 cells with a *luciferase* reporter gene under the control of the mouse *alpha glycoprotein (αGSU)* promoter **(C).** Promoter activity was assayed by measuring luciferase activity 48 h after transfection. Negative controls (Control) received equivalent amounts of empty expression vector plasmid. Activities are mean (light units/10 s/g total protein) of triplicate assays ± SEM. A representative experiment of at least three experiments is depicted. Similar experiments to those depicted in **(C)** were performed using a rat *prolactin (PRL)* promoter/enhancer reporter gene **(D)**. Adapted from (Bechtold-Dalla Pozza et al., 2012).

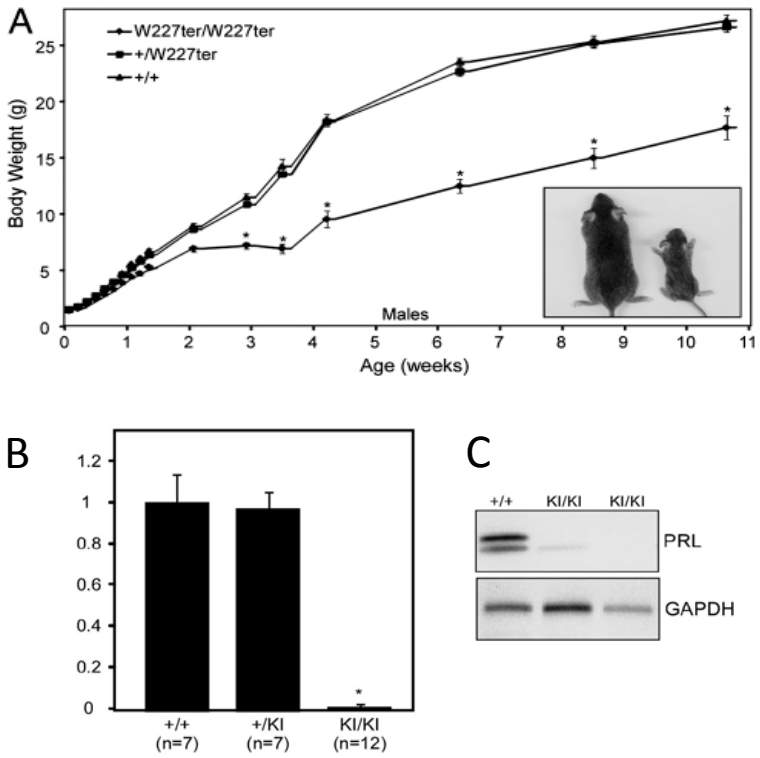

Figure 5. *Lhx3*$^{W227ter/W227ter}$ mice are dwarfed and have a PRL deficiency. (A) Growth deficiency of male homozygote *Lhx3*$^{W227ter/W227ter}$ mice becomes significant at postnatal day 21 (P21) compared to controls. Asterisks (*) indicate a significant difference between *Lhx3*$^{W227ter/W227ter}$ compared with wild-type (+/+) and heterozygote controls with $P < 0.05$ (ANOVA). Error bars are ±SEM. *Inset* shows *Lhx3*$^{+/+}$ (*Left*) and *Lhx3*$^{W227ter/W227ter}$ (*Right*) male litter mates at 8 wk old. **(B)** Real-time quantitative PCR analyses demonstrate a reduction in *Prl* transcripts in *Lhx3*$^{W227ter/W227ter}$ pituitaries (KI/KI) relative to wild-type (+/+) and heterozygote (+/KI) counterparts at 12 wks of age. Data are mRNA levels normalized to a control transcript (*36b4*). Error bars are ±SEM. Asterisk (*) indicates significance compared with wild-type controls with $P < 0.05$ or less (Student's two-tailed *T*-test). **(C)** Western blot detection of PRL protein in 12-wk pituitaries confirms the PRL deficiency in the *Lhx3*$^{W227ter/W227ter}$ mutant mice compared with wild-type controls. Blots were reprobed with an anti-GAPDH antibody as a loading control. Adapted from (Colvin et al., 2011).

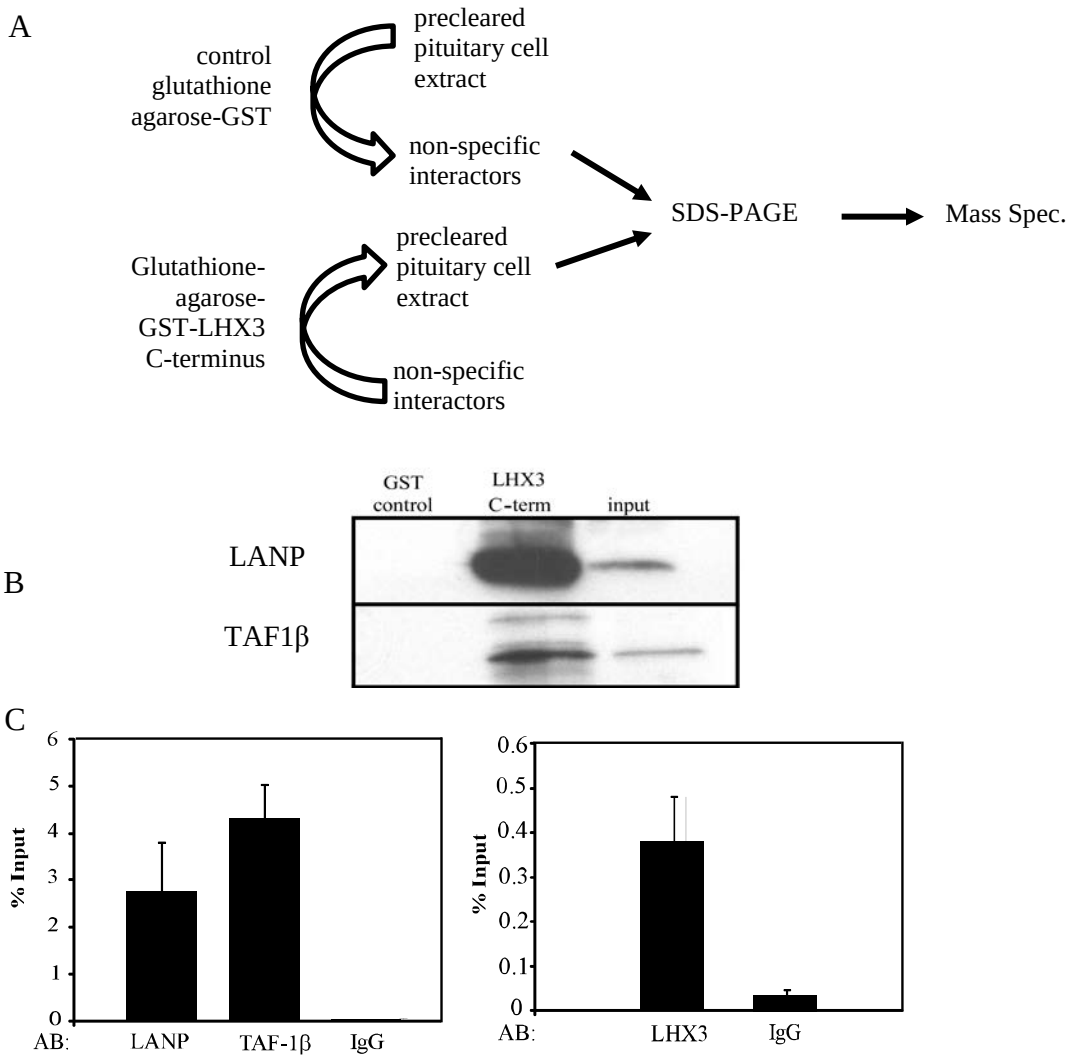

Figure 6. Characterization of the LHX3-INHAT relationship. (**A)** Affinity purification scheme for purifying LHX3 carboxyl terminus interacting proteins from cellular extracts using GST fusions bound to a solid support. GST alone and GST-LHX3 carboxyl-terminus bound proteins were compared via mass spectroscopy or western blotting. (**B)** Elutions from affinity purifications were tested by western blotting for enrichment of INHAT proteins. **(C)** The LANP and TAF-1β INHAT proteins are associated with the LHX3-bound *αGSU* gene promoter. Chromatin immunoprecipitation (ChIP) was used to probe occupancy of the pituitary glycoprotein binding element (PGBE) region of the mouse *αGSU* promoter (Roberson et al., 1994; Bach et al., 1995) by LANP, TAF-1β (left) or LHX3 (right) proteins in LβT2 pituitary cells. ChIP enrichment was measured by quantitative PCR and represented as percent input, calculated by 100*2^(input - Ct (IP)). Values are mean ± SEM for three independent experiments. Immunoprecipitation with non-immune species-matched IgG were carried out as negative controls. Adapted from Hunter/Malik et al. (submitted 2013).

Methods

1. Bioinformatic Analysis

EMBOSS CpGPlot (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) was used to determine potential CpG islands in the mouse and human *Lhx3* gene. Mouse and human *Lhx3/LHX3* gene sequences (UCSC Genome Bioinformatics, http://genome.ucsc.edu/), spanning -3.5 kb upstream and 8 kb downstream of the translation start site were used as input. The minimum length of the reported CG was 200 bp. The observed to expected ratio (Obs/Exp) of C plus G to CpG in a set of 10 windows required before a CpG island is reported was 0.6. DNAsis Software was used to analyze the *Lhx3* sequences.

2. Cell Culture

The Pit-1/0 and Pit-1/Triple cell lines (Sizova et al., 2010) a generous gift from Dr. R. Day, IUSM) were maintained in Dulbecco's Modified Eagle's Media/Ham's Nutrient Mixture F-12 (1:1) with 2.5 mM L-glutamine (Hyclone, Logan UT) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaillé, France) and antibiotic-antimycotic (Invitrogen, Gran Island, NY). The cells were grown at 37°C in humidified 5% CO_2containing atmosphere.

3. Cell Treatments

Cells ($1x10^6$ cells/10 cm) were plated and 24 h later treated with 5-aza-2'-deoxycytidine (2.5 μM 5-aza-dc, Sigma-Aldrich, St. Louis, MO) or 5-aza-dc (2.0 μM) plus Trichostatin A (100 nM, TSA) in media containing 10% FBS. Cells treated with 5-aza-dc plus TSA were harvested 24 h after treatment, while cells treated with only 5-aza-dc were harvested 48 h after treatment. Control plates were treated with DMSO (100 μl) as vehicle.

4. Bisulfite Genomic Sequencing

Genomic DNA was isolated from treated and untreated Pit-1/0 cells and Pit-1/Triple cells using a PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The bisulfite conversion was carried out with 500 ng of input genomic DNA with the use of the EZ DNA Methylation-Gold Kit™ (Zymo Research Corp., Orange, CA) following the manufacturer's protocol. Supplied Conversion Reagent (130 µl) was added to 20 µl of DNA (500 ng). The sample was subjected to thermal cycling with the following parameters per kit instructions: 98°C for 10 m, 64°C for 2.5 h and 4°C for up to 20 h. The sample was then mixed with binding buffer and applied by centrifugation to the Zymo-Spin™ ion chromatography (IC) column. The bound materials were washed and desulphonation buffer was added to the column. After incubating the column for 15 m the sample was centrifuged and washed. The bisulfite converted DNA was eluted in a volume of 10 µl RNase free water and then diluted 1:10 for a final volume of 50 µl. The bisulfite converted DNA (5 µl) was amplified by PCR using the Fast Start High Fidelity PCR System (Roche, Mannheim, Germany) with specific primers to the *Lhx3a* and *Lhx3b* promoters. Primers designed using the MethPrimer software (http:/ww.urogene.org/methprimer/) were as follows: m*Lhx3a* promoter forward, 5′- ggaaggttagagaaaaggttattt-3′; m*Lhx3a* promoter reverse, 5′-aaaaaacaaaaactccaaaaacac-3′; m*Lhx3b* promoter forward, 5′-gtatttgtaggaagttttgagtggtatt-3′; *Lhx3b* promoter reverse, 5′- ccctaaacttcccctaaatctaac-3′. A 'hot' start PCR was performed by initially heating the reaction to 95°C for 2 m, prior to the addition of enzyme. The reaction then underwent 40 cycles of 95°C for 2 m, 59°C for 30 sec., 72°C for 1 m, and a final extension time 7 m. The PCR products were gel purified using a

Qiaquick™ gel extraction kit (Qiagen, Valencia, CA), then sub-cloned into the pCR-4 TOPO vector using the TOPO™ TA Cloning kit (Invitrogen, Grand Island, NY). The plasmid was transformed according to the manufacturer's protocol. Briefly, *E.coli DH5α* cells (50 μl) and plasmid DNA (2 μl) were incubated on ice for 15 m. The reaction mixtures were heat shocked at 42°C for 45 seconds followed by incubation on ice for 2 m Super Optimal Broth (SOC, Invitrogen, Grand Island, NY) was added and the cells were incubated with shaking at 37°C for 0.5-1 h. Cells were then plated on antibiotic appropriate LB agar plates. Plasmid DNA from at least 15 unique colonies per group was prepared using PureLink™ Quick Plasmid Miniprep (Invitrogen, Grand Island, NY) and sequenced (ACGT, Inc, Wheeling, IL) using M13 Forward (-20) universal primer.

5. RNA Isolation and Reverse Transcription

Endogenous or treated Pit-1/0 cells ($1x10^6$ cells/10 cm plate) and Pit-1/Triple cells ($1x10^6$ cells/10 cm) were plated and RNA was harvested 24 h later. Total RNA was extracted using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using High Capacity Reverse Transcription kit (Applied Biosystems, Grand Island, NY) according to the manufacturer's protocol. The cDNA was used as a template for PCR reactions performed with GoTaq Green Master Mix (Promega, Madison, WI). Primers used were as follows: *mLhx3a* forward, 5′- aaccactggattagtgactg-3′; *mLhx3b* forward, 5′-gaagttcagggtcggaggg-3′; *mLhx3a/b* reverse, 5′- tggtcacagcctgcacacat-3′; *β-actin* forward, 5′-ctacaatgagctgcgtgtgg-3′; *β-actin* reverse, 5′-tagctcttctccagggagga-3′; *αGSU* forward, 5′-aatcacctgcccagaacacat-3′; *αGSU* reverse 5′-agcgcgtcagaagtctggta-3′; *TSHβ* forward 5′-tactgcctgaccatcaacac-3′; *TSHβ* reverse 5′-ttctgacagcctcgtgtatg-3′. The

PCR parameters were as follows: 95°C for 2 m, 40 cycles of 95°C for 30 sec., 55-58°C for 1 m, 72°C for 1 m, and a final extension time of 72°C for 7 m. The PCR products were analyzed on the Mini-PROTEAN 10% TBE precast gels (Biorad, Hercules, CA) by electrophoresis.

6. Protein Analysis

6.1. Protein isolation

Whole cell protein extracts of Pit-1/0 cells ($1x10^7$ cells/15 cm plate) and Pit-1/Triple cells ($1x10^7$ cells/15 cm) were isolated for Western blotting. Briefly, the cells were rinsed with cold PBS and harvested in one ml cold PBS. The cells were centrifuged for one minute at 6,000 x *g* and the PBS was removed. The cell pellet was suspended in RIPA Lysis buffer (150 mM NaCl, 2mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.01 sodium phosphate, pH 7.2, 50 mM NaFl) containing protease inhibitors (Complete Mini protease inhibitor cocktail tablet, Roche, Mannheim, Germany) and the cells were lysed on ice for 15 m After incubation, the sample was centrifuged at 13,000 x *g* for 10 m. Supernates were aliquoted and stored at -20°C. The BCA Protein Assay (Thermo Scientific, Rockford, IL) was used to determine protein concentration.

6.2. Western blot

The harvested protein (25 μg) was subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE; 12%; 100V). After electrophoresis, gels were equilibrated for 5 minutes in western transfer buffer (192 mM glycine, 2 mM Tris, 20% methanol). PVDF membranes were charged in methanol, prior to transfer. The transfer apparatus was assembled according to the manufacturer's instructions (Biorad). Protein was transferred at 80 mAmps in 4°C with an ice block. After transfer, the PVDF membrane

was washed with TBST (Tris-Buffered Saline Tween (154 mM NaCl, 52 mM Tris, 1% Tween 20) with shaking. The membranes were blocked in 5% nonfat dry milk (NFDM, Carnation) in TBST for 1 h with shaking. After blocking, the membranes were washed 3x 5 m each with TBST. Each membrane was then incubated with LHX3 (Chemicon, Temecula, CA; 1:2,500), MBD2 (Abcam, Cambridge, MA; 2 μg/μl) or MeCP2 primary antibody (Abcam; 2 μg/μl) in TBST overnight at 4°C with shaking. The membrane was washed 3x 5 m with TBST and then incubated with an appropriate peroxidase conjugated secondary antibody (1:25,000) in TBST for 1 h. Bound antibody was visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) for 1 m with gentle mixing prior to documentation by G-Box Imaging System (Syngene, Frederick, MD).

7. Chromatin Immunoprecipitation Assay

Pit-1/0 chromatin was isolated for chromatin immunoprecipitation (ChIP) analysis. Pit-1/0 cells (~1-2 x 10^7) were grown in 15 cm culture dishes and treated with 5-aza-dc (2.5 μM), 5-aza-dc (2.0 μM) plus TSA (100 nM) or DMSO vehicle (100 μl). Twenty-four hours after treatment, the chromatin was isolated from the control plate and 2.0 μM 5-aza-dc plus 100 nM TSA treated cells. Cells from the 5-aza-dc only treatment group were harvested 48 h post treatment. ChIP assays were carried out with the EZ ChIP Kit (Millipore, Billierica, MA) following the manufacturer's protocol. Each 15 cm dish of Pit-1/0 cells was washed twice with filtered phosphate buffered saline (PBS) The chromatin was crosslinked with 1% formaldehyde (Thermo Scientific, 16% Stock) prepared in PBS for 8 min at room temperature. The crosslinking reaction was terminated with the addition of glycine (2.5 μM) followed by a 5 m incubation at room temperature.

The media was aspirated and cell monolayers were washed two times with cold PBS. The cells were then quantitatively transferred in cold PBS (1.0 ml) to a 1.5 ml tube on ice and centrifuged at 4,000 x *g* for 5 m at 4°C. Cell pellets were resuspended in SDS Lysis Buffer (200 µl/ per 1 million cells), supplemented with PMSF and incubated on ice for 10m. The lystes were subjected to sonication (3 x 11 m; on 30 s, off 30 s) using a water bath sonicator. After sonication, the samples were centrifuged at 13,000 x *g* for 10 m at 4°C and the supernates were aliquoted (200 µl). A 20 µl aliquot was used to check for shearing efficiency. Briefly, 30 µl of TE was added to the 20 µl aliquot for a total volume of 50 µl. Crosslinks were reversed by adding 5 M NaCl (2 µl) to the sample and incubating it at 65°C overnight. The sample was treated with RNase (1 µl; 10mg/ml) for 15 m at 37°C. To remove proteins, Proteinase K (1 µl; 10 mg/ml) was added and the sample was incubated at 45°C for 1-2 h The sheared samples were analyzed on 1.5% agarose gel to check the generation of DNA fragments (<1 kb).

Chromatin isolated from Pit-1/0 cells (200 µl) was mixed with cold dilution buffer containing protease inhibitors. The samples were precleared for 1 h with Salmon Sperm DNA Blocked Protein A agarose beads (60 µl, Millipore). An input control sample was removed (1%) from the precleared chromatin and stored at 4°C for downstream PCR analysis. Supernates were incubated with antibody (5 µg) overnight with gentle shaking. The following antibodies were used: anti-acetyl Histone 3 (Millipore, Temecula, CA), MeCP2 (Abcam, Cambridge, MA), MBD2 (Abcam) and rabbit IgG as a negative control. Following the overnight incubation, Protein A beads (60 µl) were added and the samples were incubated at 4°C for 2-4 h. In accordance with the manufacturer's protocol, the beads were washed for 5 m with 1 ml of each solution provided in the kit in the order

listed at 4°C: low salt buffer, high salt buffer, LiCl buffer and two washes with TE. The sample was eluted with elution buffer containing SDS and $NaHCO_3$ and purified using columns provided in the ChIP kit.

Relative abundance of regions of interest in precipitated DNA was measured by quantitative PCR (qPCR) (ABI 7900 PRISM, Applied Biosystems, Foster City, CA) using SYBR green (Roche). The isolated ChIP input DNA was diluted 1:10 and primers to the *Lhx3* promoter were as follows: *Lhx3a* forward, 5'-tgaaagaggtccagcacttcc-3'; *Lhx3a* reverse, 5'-tggcaatcgagttctgcttc-3'; *Lhx3b* forward, 5'-tctgtaggaagccttgagtgg-3'; *Lhx3b* reverse, 5'-cgtctggcttgcacttcc-3'. The PCR parameters were as follows: 95°C 15 m for one cycle, 95°C 15 sec, 60°C 60 sec for 40 cycles and 95°C 15 sec, 60°C 1 m, 95°C 15 sec for one cycle. Fold enrichment was calculated using the following formula: $2^{\wedge-[Ct(IgG) - Ct-(sample)]}$

Results

1. Analysis of *Lhx3* mRNA and LHX3 Protein Expression

Recently, mouse cell lines representing different stages of anterior pituitary cell development have been generated using pituitary promoters to target immortalizing genes in transgenic animals (Sizova et al., 2010). Of these, the Pit-1/0 and Pit-1/Triple lines represent earlier and later developmental stages, respectively. Unfortunately, similar reagents modeling human pituitary cell types are not available. To examine whether the *Lhx3a* and *Lhx3b* mRNAs are expressed in Pit-1/0 and Pit-1/Triple cells, total RNA was isolated and *Lhx3a*, *Lhx3b* and *actin* (positive control) transcripts were detected by RT-PCR. The Pit-1/Triple cells expressed both *Lhx3a* and *Lhx3b*, while neither transcript was expressed in the Pit-1/0 cells (Figure 7A). Correspondingly, LHX3 protein was detected in the Pit-1/Triple cells, but was not observed in the Pit-1/0 cells using western assays of whole cell protein extracts (Figure 7B).

2. Identification and Investigation of Methylated Regions in *Lhx3* Promoters

To determine if methylation might play a role in *Lhx3* gene expression, bioinformatics was used to identify possible CpG-rich regions in the *Lhx3* promoters. The EMBOSS CpGPlot online tool predicted a CpG-rich region encompassing the *Lhx3b* promoter of both the mouse and human genes. A CpG-rich region also was noted in the human *LHX3a* promoter, but the corresponding region in the mouse *Lhx3a* gene promoter does not have quite as high a CpG density. To further probe the role of methylation of the two mouse *Lhx3* promoters, a 333 bp region of the mouse *Lhx3a* promoter (nucleotides -166 to +166 relative to the transcription start site) containing SP1 binding sites and 15 CpG sites was identified for further analysis (Figure 8A). Similarly, the CpG- rich region

corresponding to the mouse *Lhx3b* promoter was covered by a 321 bp region (nucleotides -235 to +85) containing 30 CG sites and SP1 binding sites A (Figure 9A).

In order to characterize the methylation status of the *Lhx3* promoters in the Pit-1/0 cell line, genomic DNA was subjected to bisulfite conversion and sequencing. During bisulfite conversion, cytosines (C) are converted to thymines (T), but 5-methylcytosines (5mC) remain unaltered. Bisulfite sequencing from a series of individual DNA clones extracted from Pit-1/0 cells demonstrated that the *Lhx3b* promoter is highly methylated, while the *Lhx3a* promoter is less methylated (Figure 8B and 9B). Interestingly, the more differentiated Pit-1/Triple cells were less methylated in the central region compared to the precursor Pit-1/0 cells (Figure 8C and 9C).

3. 5-Aza-2'-deoxycytidine Induces *Lhx3* Gene Expression

To determine whether *Lhx3* promoter methylation could be linked to the lack of detectable expression in Pit-1/0 cells, the cells were treated with the demethylating reagent, 5-Aza-2'-deoxycytidine (5-aza-dc). *Lhx3a* expression was induced 24 hours after 2.5 μM 5-aza-dc treatment, and both transcripts were expressed 48 hours post treatment (Figure 10A), suggesting that methylation plays a role in *Lhx3* expression. Interestingly, when the demethylating reagent was removed after 24 hours and the mRNA was harvested 48 hours after initial treatment, *Lhx3* expression was repressed, consistent with methylation being a dynamic process (Figure 10B). Bisulfite sequencing of individual DNA clones from Pit-1/0 cells treated with 5-aza-dc for 48 hours (higher level of *Lhx3* transcripts) showed a decrease in methylation at specific individual sites of both the *Lhx3a* and *Lhx3b* promoters (Figure 11A, B). The CpG sites at +12 and +17 of the *Lhx3b*

promoter were not as affected by the treatment, with 57% and 50% of the clones methylated at these positions, respectively.

4. Trichostatin A and 5-aza-dc Treatment Induce *Lhx3* Expression

DNA methylation has been shown to recruit chromatin modifying proteins to modulate gene expression (reviewed in (Berger, 2002; Cosgrove and Wolberger, 2005; Vaissiere et al., 2008; Bogdanovic and Veenstra, 2009; Zhang and Ho, 2011; Buck-Koehntop and Defossez, 2013)). To investigate whether the interaction between DNA methylation and chromatin modification could alter *Lhx3* expression, Pit-1/0 cells were treated with both 5-aza-dc and the HDAC inhibitor, trichostatin A (TSA). The optimal concentration of 5-aza-dc plus TSA for *Lhx3* gene expression was determined to be 2.0 μM 5-aza-dc plus 100 nM TSA for 24 hours (Figure 12), as the 2.5 μM 5-aza-dc plus 100 nM TSA treatment killed the cells. The combined treatment induced expression of both *Lhx3* transcripts (Figure 13A). Bisulfite sequencing of the Pit-1/0 cells treated with 5-aza-dc plus TSA indicated decreased methylation at specific individual sites of both *Lhx3* promoters (Figure 13B, C). Treatment with TSA plus 5-aza-dc notably decreased methylation at the CpG sites +12, +17, +22, +27, +38 and +52 of the *Lhx3b* promoter compared to the bisulfite sequencing of untreated Pit-1/0 cells. In dual treated cells, bisulfite sequencing revealed that although methylation decreased at CpG sites -206 and -197 of the *Lhx3b* promoter, 50% or higher of clones still remained methylated after treatment.

5. Inhibitor treatment did not induce *αGSU* or *TSHβ* gene expression

To determine if inhibitor treatment induced hormone gene transcription, mRNA was harvested from treated and untreated Pit-1/0 cells and regions of the *αGSU* and *TSHβ*

genes were amplified by RT-PCR. Treatment of Pit-1/0 cells with 5-aza-dc or 5-aza-dc plus TSA did not induce transcription of *αGSU* or *TSHβ.* Pit-1/Triple cells were used as a positive control for *αGSU* and *TSHβ* expression (Figure 14).

6. The MeCP2 Methyl-Binding Protein Occupies *Lhx3* Promoters

Methyl-CpG-binding proteins bind methylated DNA to regulate gene transcription (Robertson and Jones, 2000; Bogdanovic and Veenstra, 2009). MeCP2, a member of the methyl-binding domain (MBD) family of proteins is a regulator of global *de novo* methylation and has been shown to bind the pituitary *proopiomelanocortin* (*Pomc)* gene promoter (Tao et al., 2009). We determined if MeCP2 and its related protein family member, MBD2 were expressed in the Pit-1/0 pituitary cell line. Whole cell extracts from Pit-1/0 cells were subjected to Western analyses using antibodies recognizing MeCP2, MBD2 and GAPDH (positive control). Both MeCP2 and MBD2 protein were detected in the Pit-1/0 cells (Figure 15A). Because treatment with the demethylating reagent, 5-aza-dc plus the HDAC inhibitor, TSA induced *Lhx3* expression, we further investigated how the combined inhibitor treatment might alter the *Lhx3* promoter occupation by MeCP2 and acetylated histone 3 (acH3). Acetylation of histones relaxes the chromatin structure and histone acetylation is often used as a marker of gene activation (Berger, 2002). To examine the effects of the inhibitors, chromatin immunoprecipitation (ChIP) assays were performed in treated and untreated Pit-1/0 cells with antibodies to MeCP2, MBD2, acH3 and IgG (negative control) and primers to the *Lhx3* promoters. The 5-aza-dc plus TSA and control plates were harvested 24 hours post treatment and the 5-aza-dc treated cells were harvested for chromatin 48 hours post treatment to capture maximized *Lhx3* expression in the cells. Analysis of the ChIP assay

with Pit-1/0 cells treated with 5-aza-dc showed a ~five-fold decrease in acH3 occupation and a 1.2-fold decrease in MeCP2 occupation of the *Lhx3a* promoter (Figure 15B). For the *Lhx3b* promoter, 5-aza-dc treatment led to an approximately 1.5 fold increase in acH3 occupation and no change in MeCP2 occupation compared to the respective antibody in untreated cells (Figure 15C). Analysis of ChIP assays with Pit-1/0 cells treated with 5-aza-dc plus TSA indicated a ~6-fold increase in acH3 occupation and approximately a 2.4-fold decrease in MeCP2 occupation at the *Lhx3a* promoter region compared to the respective antibody in untreated cells (Figure 15B). Occupation of acH3 at the *Lhx3b* promoter of the double treated cells increased about 20-fold, while there was no major change in MeCP2 occupation of the *Lhx3b* promoter, compared to the respective antibody in untreated cells (Figure 15C). MBD2 does not appear to be associated with the *Lhx3* promoters (Figure 15B and C). A region within the *Lhx3* gene was amplified as a negative control to show MeCP2 antibody specificity (Figure 15D).

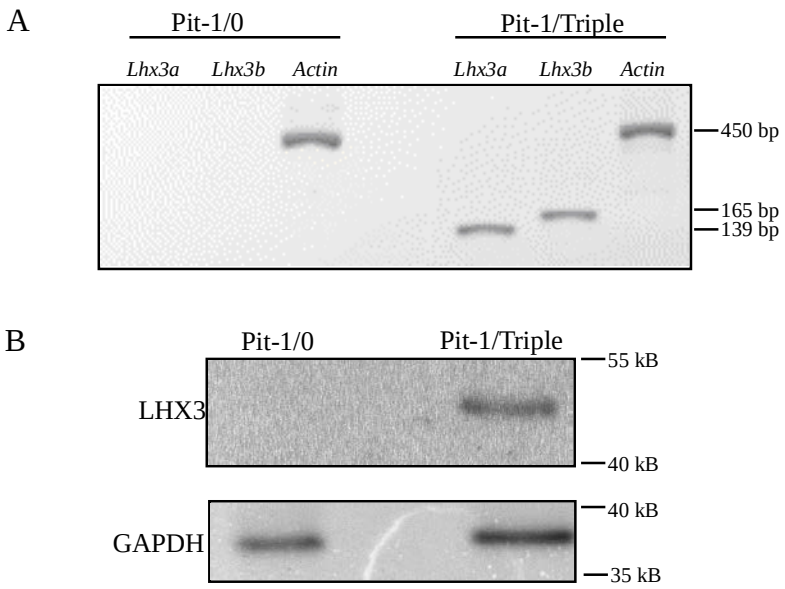

Figure 7. *Lhx3* mRNA and LHX3 protein are expressed in Pit-1/Triple, but not Pit-1/0 cells. (**A**) *Lhx3a* and *Lhx3b* mRNA was amplified by RT-PCR in Pit-1/0 and Pit-1/Triple cells. *Beta actin* primers were used in a positive control to test cDNA integrity. **(B)** Protein levels of LHX3 were determined by western blotting of whole cell protein extracts of Pit-1/0 and Pit-1/Triple cells, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. Each experiment was performed three times with similar results.

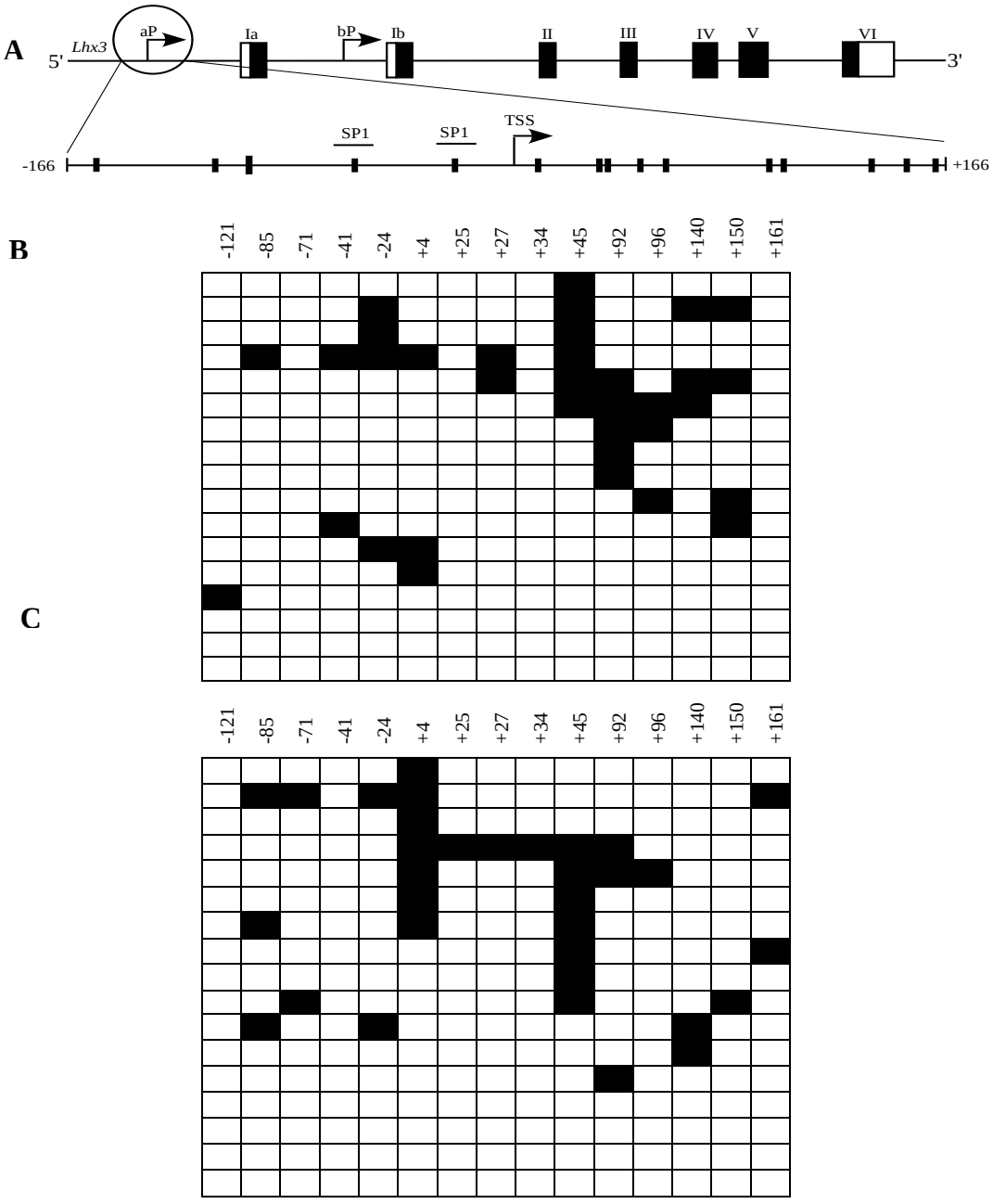

Figure 8. *Lhx3a* promoter and its methylation pattern. (A) The upper diagram represents the mouse *Lhx3* gene. Black rectangles = protein-coding exons; white areas = untranslated exonic regions. Two upstream promoters (aP and bP) produce the *Lhx3a* and *Lhx3b* mRNAs. The lower diagram methylation sites in the *Lhx3a* promoter (-166 to +166m, with the transcription start site (TSS) set to position 0). Vertical black bar = CG base pair; SP1 binding sites shown (Yaden et al., 2006). **(B)** The methylation pattern of the *Lhx3a* promoter in mouse Pit-1/0 cells and *Lhx3a* promoter in mouse Pit-1/Triple cells **(C)** was determined by bisulfite sequencing (methods). Black boxes = methylated CpGs and open boxes = non-methylated CpGs.

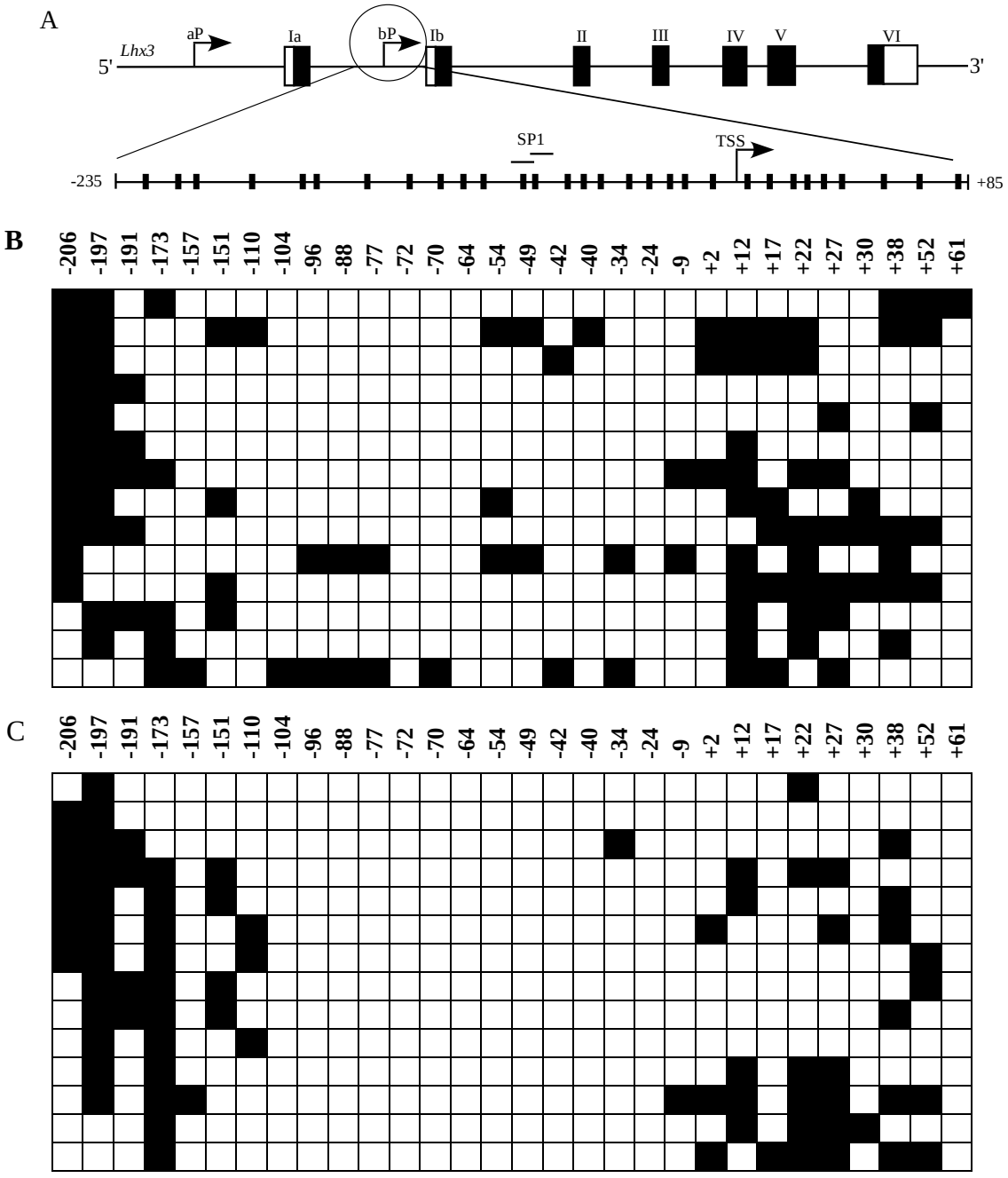

Figure 9. *Lhx3b* promoter and its methylation pattern. (A) The upper diagram represents the *Lhx3b* gene, with black rectangles signifying protein-coding exons and white areas signifying untranslated exonic regions. Two upstream promoters (aP and bP) produce the *Lhx3a* and *Lhx3b* mRNAs. The diagram below shows sites of methylation in the *Lhx3b* promoter (-235 to +85 with the transcription start site (TSS) set at position 0). Each vertical black = CG base pair. SP1 binding sites are shown. **(B)** The methylation pattern of the *Lhx3b* promoter in mouse Pit-1/0 cells and *Lhx3b* promoter in mouse Pit-1/Triple cells **(C)** was determined by bisulfite sequencing (methods). Black boxes = methylated CpGs and open boxes = non-methylated CpGs.

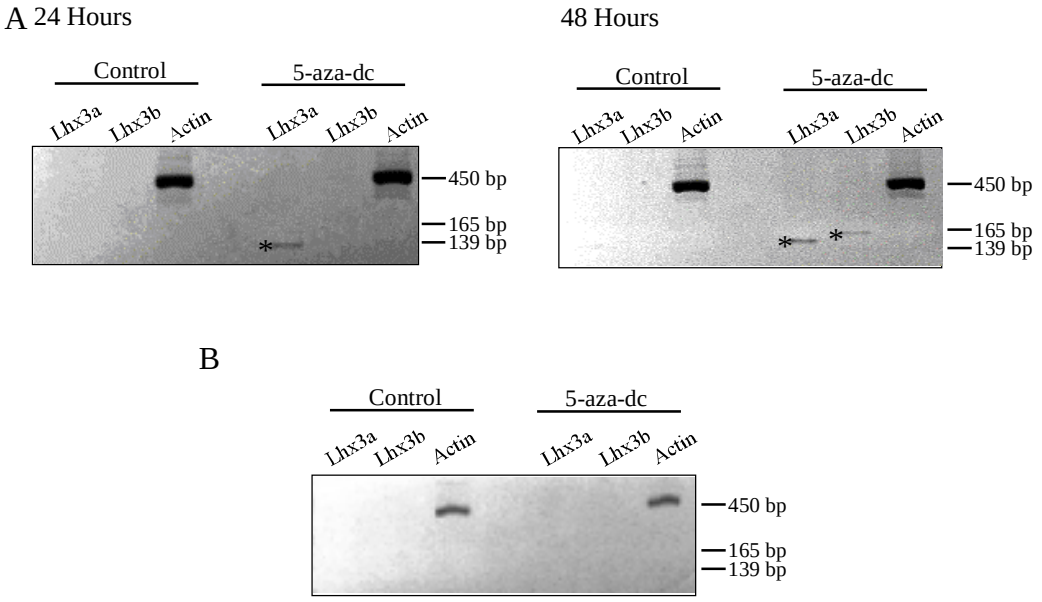

Figure 10. *Lhx3* gene expression is activated in mouse Pit-1/0 cells after treatment with 5'-aza-dc. (A) Pit-1/0 cells were treated with 2.5 μM 5-aza-dc and RNA was harvested 24 hours (left) or 48 hours (right) after treatment and RT-PCR was performed. **(B)** *Lhx3* mRNA expression post removal of 5-aza-dc. Pit-1/0 cells were treated with 2.5 μM 5-aza-dc, washed 24 hours later and mRNA was harvested 48 hours post treatment for RT-PCR analysis.

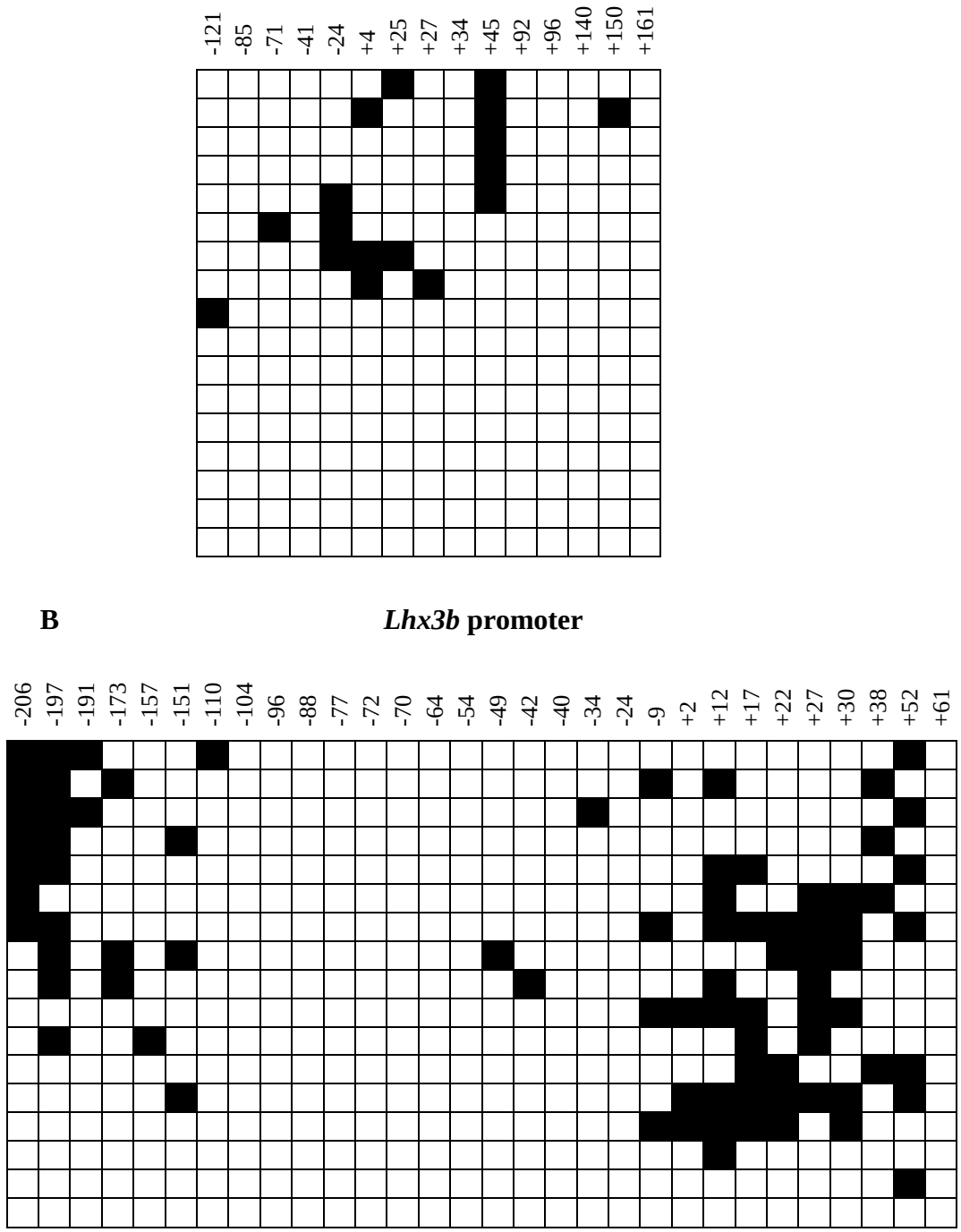

Figure 11. Bisulfite sequencing of the *Lhx3* promoters post 5-aza-dc treatment. The methylation pattern of the *Lhx3a* **(A)** and *Lhx3b* **(B)** promoters were determined by bisulfite sequencing as described in the methods. Black boxes indicate methylated CpGs and open boxes indicate non-methylated CpGs.

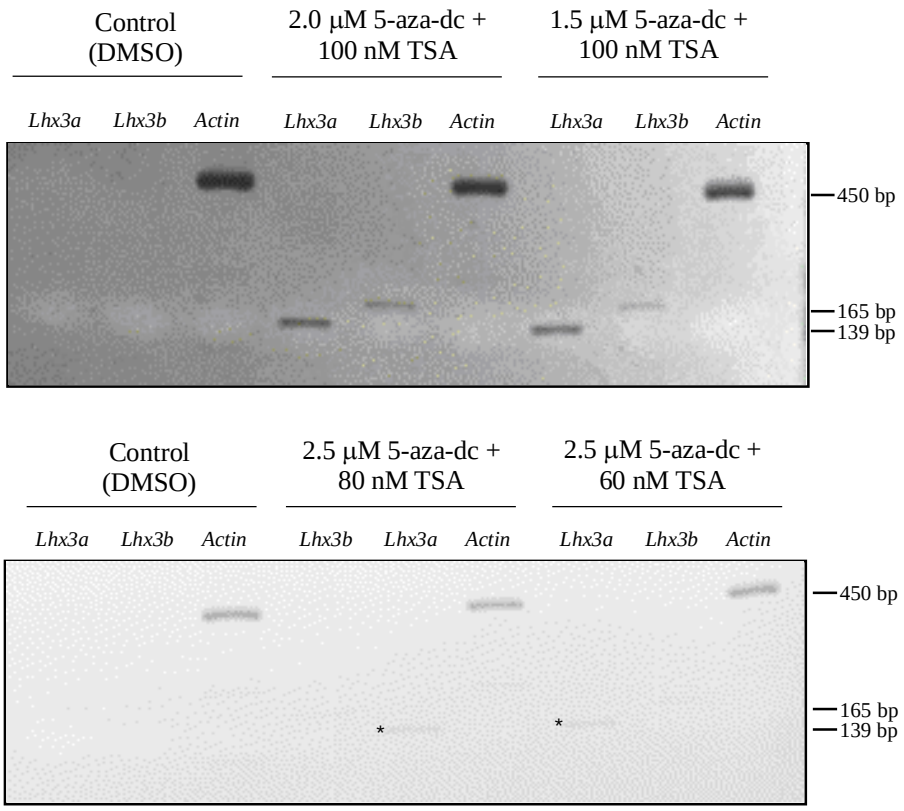

Figure 12. *Lhx3* gene expression level varies with concentration of 5-aza-dc plus TSA. Pit-1/0 cells were treated with varying concentrations of 5-aza-dc plus TSA and mRNA was harvested 24 hours post treatment for RT-PCR analysis.

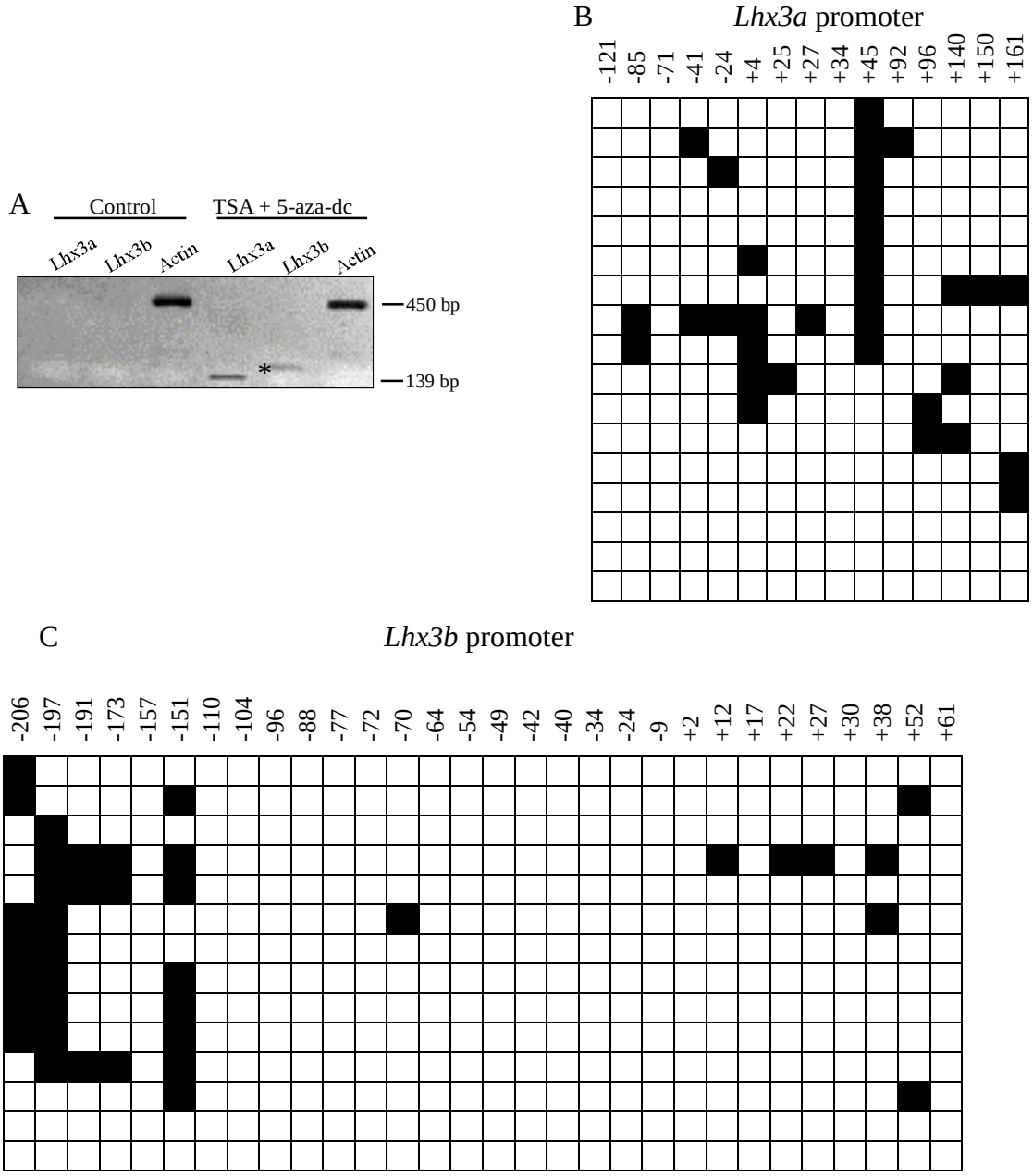

Figure 13. *Lhx3* expression is activated after treatment with 5-aza-dc plus TSA in Pit-1/0 cells. (**A)** Mouse Pit-1/0 cells were treated with 2.0 μM 5-aza-dc plus 100 nM TSA and harvested 24 hours after treatment for RNA. Pit-1/0 cells were treated with 2.0 μM 5-aza-dc plus 100 nM TSA and genomic DNA was harvested for bisulfite conversion analysis at 24 hours post treatment. The methylation pattern of the *Lhx3a* **(A)** and *Lhx3b* **(B)** promoters were determined by bisulfite sequencing as described in the methods. Black boxes indicate methylated CpGs and open boxes indicate non-methylated CpGs.

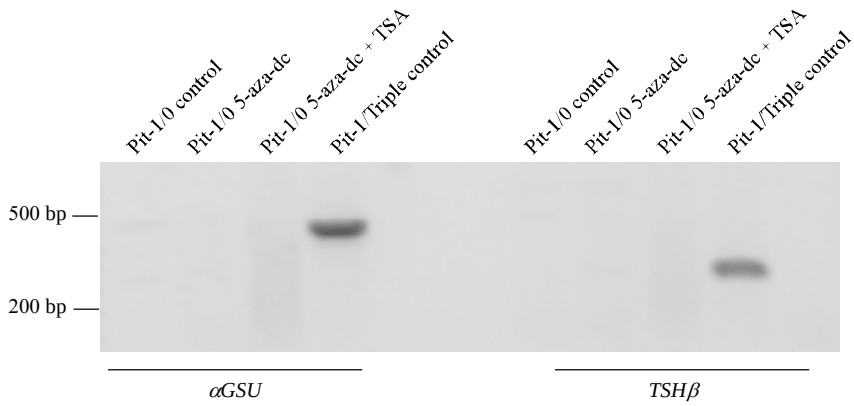

Figure 14. *αGSU* and *TSHβ* hormone transcripts are not activated in treated Pit-1/0 cells. Pit-1/0 cells were treated with 5-aza-dc or 5-aza-dc plus TSA. Hormone transcripts were amplified by RT-PCR (methods).

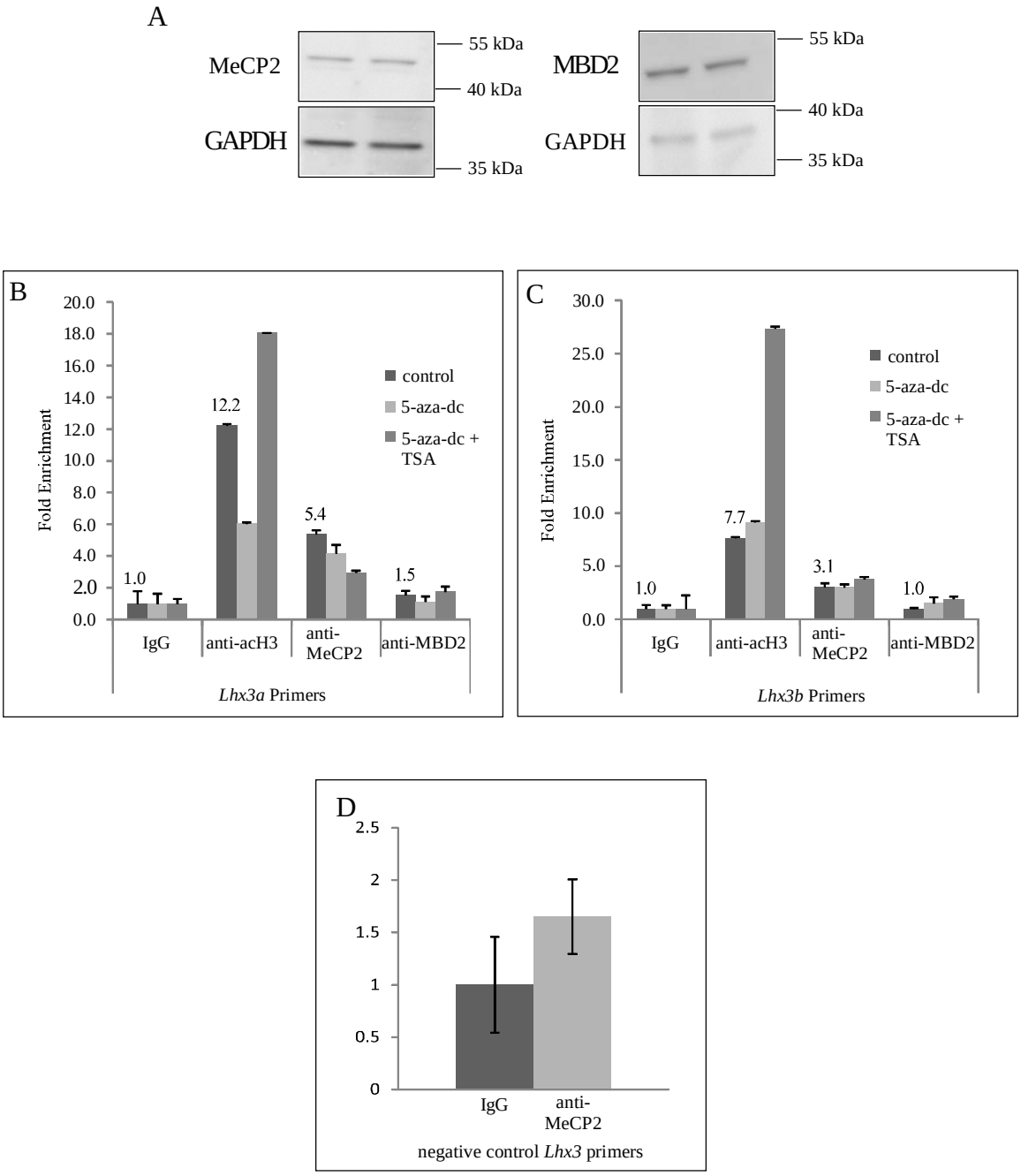

Figure 15. MeCP2 binds the *Lhx3* gene in Pit-1/0 cells. (A) Protein levels of MeCP2 and MBD2 were determined by western blotting, with GAPDH as a loading control. **(B, C)** ChIP assay was carried out using anti-MeCP2, anti-MBD2, anti-acetylated histone H3 (acH3) and anti-IgG (negative control) and primers to the *Lhx3a* and *Lhx3b* promoters. Each experiment was performed three times with similar results. **(D)** ChIP assay was carried out using anti-MeCP2 and IgG with primers to a region of the *Lhx3* gene to show binding specificity of anti-MeCP2 antibody.

Discussion

The anterior pituitary gland secretes hormones to regulate key physiological processes such as metabolism, growth, lactation, reproduction and stress response. The development of the hormone-secreting cell types is the result of coordinated signaling and interactions between key regulatory factors. One contributing transcription factor is the LIM-homeodomain protein, LHX3, which plays a critical role in the formation of somatotropes, lactotropes, gonadotropes and thyrotropes— four of the five main hormone-secreting anterior pituitary cell types. The functions of LHX3 in modulating pituitary gene expression have been extensively studied by both our lab and others; however, the molecular mechanisms controlling and regulating its expression are not fully understood. Therefore, the focus of this thesis was to determine if DNA methylation, a well-characterized process of gene control, contributed to the regulation of the *Lhx3* gene.

DNA methylation has roles in a variety of cellular processes, including transcriptional regulation (Bird, 1992). Methylation occurs at the fifth position of a cytosine residue (5mC) in the CpG context, so GC-rich DNA regions can be modified by DNA methylation (Smith and Meissner, 2013). The *Lhx3* gene has two TATA-less, GC-rich promoters and recent studies have demonstrated methylated CpG islands in the promoters of developmental genes, such as the *Hox* genes (Auclair and Weber, 2012). Based on these findings, it was hypothesized that DNA methylation patterns may affect *Lhx3* gene expression. This dissertation describes the first investigation of the role of DNA methylation in regulating *Lhx3* gene transcription.

The recently established murine Pit-1/0 and Pit-1/Triple cell lines represent different phases of PIT-1- dependent cell differentiation in the mouse anterior pituitary

(Sizova et al., 2010). As described above, PIT-1 is a POU-homeodomain protein that is involved in the coordination and differentiation of somatotropes, lactotropes and thyrotrope cells of the anterior pituitary, and it can act synergistically with LHX3 to regulate pituitary genes, such as *Prl* (reviewed by (Savage et al., 2003)). In the Pit-1/0 cells, the *Pit-1* gene is expressed, but the cells fail to express PIT-1-dependent hormones. The Pit-1/Triple, representing a later phase of differentiation, express the *Pit-1* gene, as well as the *GH*, *PRL* and *TSHβ* hormone genes. The expression of the *Lhx3* gene in these "Pit-1 lineage" cell lines had yet to be determined. In this study, we found that the Pit-1/0 cells did not express either the *Lhx3* gene or the LHX3 protein, whereas the Pit-1/Triple cells produce LHX3. Therefore, the Pit-1/0 cells represent a valuable model to investigate the role of DNA methylation in *Lhx3* gene expression.

Bioinformatics was used to identify corresponding CpG-rich regions in the mouse and human *Lhx3* promoters for further analysis. CpG-rich regions in the *Lhx3a* and *Lhx3b* promoters contained two previously identified SP1 binding sites (Yaden et al., 2006). SP1 has established roles in basal transcriptional activity of genes with GC-rich promoters, and SP1 binding are known as sites of DNA methylation control in some cases (Li et al., 2004). Although there are multiple studies examining the methylation status of SP1 binding sites, the findings are conflicting. Some studies have found that methylation of SP1 binding sites prevents its binding and subsequent gene activation, while others have shown that methylation of SP1 binding sites does not alter its binding (e.g. (Harrington et al., 1988; Holler et al., 1988; Mancini et al., 1999; Butta et al., 2006; Douet et al., 2007)). We found that there was no obvious difference in methylation patterns associated with SP1 binding sites between the Pit-1/0 cell lines, which do not

express the *Lhx3* gene, and the Pit-1/Triple cell line that does express the *Lhx3* gene. Our findings suggest that the methylation status of the promoter SP1 sites is not critical for the transcriptional status of the *Lhx3* gene.

To further determine how DNA methylation may impact the expression of the *Lhx3* gene, we treated the cells with the demethylating reagent, 5-aza-dc. The reagent inhibits DNA methyltransferase activity, causing genome-wide hypomethylation. Treatment with 5-aza-dc induced expression of both *Lhx3* mRNA transcripts in the Pit-1/0 cells, with increased expression correlating with longer exposure to the reagent. These results are similar to various other studies that have used 5-aza-dc to induce gene expression (Bovenzi and Momparler, 2001; Glait-Santar and Benayahu, 2011; Hansberg-Pastor et al., 2013). A series of DNA clones from treated cells was subjected to bisulfite sequencing, and results showed a decrease in methylation at both the *Lhx3a* and the *Lhx3b* promoter CpG regions. Together, this data suggest that methylation may be part of *Lhx3* gene inactivity in Pit-1/0 cells.

The methylation process was further examined by removing the demethylating reagent and then assaying for *Lhx3* gene expression 24 hours post removal. Removal of the 5-aza-dc demethylating reagent led to silencing of the *Lhx3* gene within 24 hours. The mechanism of 5-aza-dc and its analog 5-azacytidine in blocking methylation have been studied in cultured cells (Creusot et al., 1982; Taylor and Jones, 1982; Christman et al., 1983). DNA methyltransferases irreversibly interact with 5-aza-dc and its analogs, forming adducts that if not repaired, become mutagenic and cytotoxic (Creusot et al., 1982; Taylor and Jones, 1982; Christman et al., 1983; Jackson-Grusby et al., 1997). Additionally, it has been found that DNMT1 becomes deactivated after azacytidine is

incorporated into the DNA, resulting in passive loss of DNA methylation a few hours after treatment (Creusot et al., 1982). A similar mechanism may be occurring in the Pit-1/0 cells, in which DNMT1 binds to a target hemi-methylated DNA template strand and irreversibly binds the incorporated 5-aza-dc on the nascent strand to form an adduct that prevents DNA methylation. Because the cells are treated with a low dose of 5-aza-dc, there may be a population of methylated and unmethylated DNA in the cell, which would explain the amplification of the *Lhx3* gene. Removal of the 5-aza-dc may give the cell time to repair the adducts, prevent new adducts from forming and properly replicate the existing methylated DNA. The population of unmethylated DNA may not be high enough for amplification by PCR.

Acetylation is a well characterized post-translational histone modification that is often a marker of gene activation (reviewed in (Berger, 2002)). The addition of an acetyl group is catalyzed by histone acetyl transferases (HATs), whereas the removal of acetyl groups is catalyzed by histone deacetylases (HDACs). Furthermore, it has been shown that silenced genes can be re-activated by treatment with 5-aza-dc plus TSA (Cameron et al., 1999). Interestingly, treatment of Pit-1/0 cells with both 5-aza-dc and the HDAC inhibitor, TSA led to induction of both *Lhx3* mRNA transcripts, suggesting possible cross-talk between the DNA methylation and chromatin microenvironment. Similar results have been seen in other studies, involving 5-aza-dc and TSA treatment to induce gene activity. For example, 5-aza-dc weakly activated expression of the *retinoic acid reporter* and *estrogen receptor α* genes in MDA-MB-231 breast cancer carcinoma cells, while the combination treatment of 5-aza-dc plus TSA resulted in greater activation of both genes (Bovenzi and Momparler, 2001).

Methyl-CpG binding proteins recruit histone modifiers to modulate transcription, so communication between methyl-CpG binding proteins and chromatin modifiers may influence *Lhx3* mRNA expression. The methyl-binding protein, MeCP2 has been shown to bind the pituitary *Pomc* gene promoter (Tao et al., 2009), and we therefore hypothesized that MeCP2 may be similarly associated with the methylated *Lhx3* promoters. ChIP assay analysis indicated that the MeCP2 is associated with both *Lhx3* promoters in Pit-1/0 cells. MeCP2 has recently been shown to interact with AT-rich DNA, through AT-hook domains (Baker et al., 2013). The *Lhx3* pituitary enhancer, Core R3 is AT-rich, so it may be possible that MeCP2 mediates the interaction between the *Lhx3* promoter and its enhancer. In this model, MeCP2 would serve as a co-activator of the *Lhx3* gene, with its function potentially determined by both *cis*- and *trans*-regulatory elements.

The C-terminal transcriptional repression domain (TRD) of MeCP2 has been shown to recruit histone deacetylases to modulate gene expression (Jones et al., 1998; Nan et al., 1998). Therefore, we performed ChIP assays to investigate whether inhibiting HDACs and/or blocking methylation would alter MeCP2 and acetylated histone H3 (acH3) occupation of the *Lhx3* promoters. Interestingly, the treatments resulted in different effects on each *Lhx3* promoter. Analysis of the ChIP assay with Pit-1/0 cells treated with 5-aza-dc showed a decrease in both acH3 occupation and MeCP2 occupation of the *Lhx3a* promoter. The decrease is MeCP2 occupation is expected, since the reagent blocks methylation and thereby potentially prevents MeCP2 from binding the methylated DNA. The decrease in acH3 occupation of the *Lhx3a* promoter with 5-aza-dc treatment suggests potential cross-talk between chromatin status and DNA methylation. One

possible explanation for the decrease is acH3 occupation of the promoter is that MeCP2 also interacts with chromatin modifying proteins to activate genes (Jones et al., 1998; Nan et al., 1998; Lunyak et al., 2002; Chen et al., 2013). For example, a study showed that MeCP2 recruits CREB1 to activate the murine neuronal *glucose transporter isoform 3* (*Glut3*) gene in murine neurons (Chen et al., 2013). Therefore, blocking methylation with 5-aza-dc prevented MeCP2 from binding and recruiting other proteins that could potentially increase or maintain basal histone acetylation at the promoter.

At the *Lhx3b* promoter, 5-aza-dc treatment led to an increase in acH3 occupation and no change in MeCP2 occupation compared to untreated cells. Because MeCP2 recruits HDACs, blocking methylation may prevent binding of MeCP2 to methylated DNA and subsequent recruitment of HDACs to histones. The absence of recruited HDACs to histones may translate to a decrease is histone deacetylation and possibly an increase in acetylation of histone H3. The lack of change in *Lhx3b* promoter occupation by MeCP2 may be due to the fact that some sites still remain methylated in the *Lhx3b* promoter after treatment and therefore may facilitate the binding of MeCP2 to the promoter. Additionally, the resolution of the ChIP assay is limited by sonication of DNA fragments less than 1 kb, so determining precise resolution of changes in MeCP2 occupation may be challenging; nonetheless, the examined sequence is within a key regulatory region of the *Lhx3* gene.

Unlike ChIP assays performed with the 5-aza-dc treatment, analysis of ChIP assays performed with Pit-1/0 cells treated with 5-aza-dc plus TSA indicated an increase in acH3 occupation and a decrease in MeCP2 occupation at the *Lhx3a* promoter region compared to the respective antibody in untreated cells. These results suggest cross-talk

and possible synergy between methyl-binding proteins and chromatin modifying proteins in regulating gene transcription, which has been seen in other studies (Jones et al., 1998; Nan et al., 1998; Klose and Bird, 2004). An increase in occupation of acH3 at the *Lhx3b* promoter resulted from the 5-aza-dc plus TSA treatment, while there was no major change in MeCP2 occupation of the *Lhx3b* promoter, compared to untreated cells. Again, the lack of change in MeCP2 occupation may be due to the remaining methylated sites after treatment.

Transcription is a dynamic process. Modifying epigenetic marks of the genes in a cell by treatment with HDAC inhibitors and/or demethylating reagents may alter the temporal expression of some genes, which in turn may result in downstream changes to both coordinated protein-protein interactions and protein-DNA interactions. Therefore, expression of the *Lhx3* gene induced by treatment with the HDAC inhibitor and/or the demethylating reagent is most likely the result of both direct and indirect effects. The change in methylation patterns after treatment (demonstrated by the *Lhx3* bisulfite sequencing) and the subsequent induction of the *Lhx3* gene suggest a direct effect, but it should be noted that other untested regions of the gene probably contributed to its expression, as well. Furthermore, the transcription of other genes that regulate *Lhx3* may have been altered by the inhibitor treatment, contributing to the indirect induction of the *Lhx3* gene. Interestingly, treatment of Pit-1/0 cells with 5-aza-dc and 5-aza-dc plus TSA did not lead to induction of the *αGSU* or *TSHβ* genes, which are downstream LHX3 and PIT-1 target genes. Additional actions therefore are required to achieve a differentiated derivative of Pit-1/0 cells that express these hormone components. While this observation indicates gene expression specificity after the cell treatments (i.e. it demonstrates that the

treatments do not generically activate many other pituitary-expressed genes in addition to *Lhx3*), the lack of hormone transcript expression may also be the result of indirect effects. For example, treating the Pit-1/0 cells with inhibitors may have induced expression of a *αGSU* or *TSHβ* repressor gene. It should also be mentioned that LHX3 protein levels in treated cells were not assayed, so it is possible that the *Lhx3* transcripts were not translated and therefore there is not LHX3 protein to interact with partners such as PIT-1 to induce the expression of hormone genes such as *αGSU* or *TSHβ*.

Collectively, these findings implicate DNA methylation in the regulation of mouse *Lhx3* gene transcription. We have shown that the demethylating reagent, 5-aza-dc can induce *Lhx3* gene activation both independently, and in combination with the HDAC inhibitor, TSA, in a pituitary precursor cell, suggesting crosstalk between DNA methylation and chromatin modifiers. The methyl-binding protein, MeCP2 has been shown to occupy the *Lhx3* promoter in Pit-1/0 cells and it may function as either a transcriptional repressor or activator, depending on the specific chromatin architecture or cellular environment.

Further studies still need to be performed to fully understand the role of DNA methylation in *Lhx3* gene regulation. This study only examined the activation of *Lhx3* in the murine pituitary Pit-1/0 cells, so similar studies could be performed in other pituitary cell lines that do not express the *Lhx3* gene, such as pituitary pre-somatotrope cell line GHFT1 (Sloop et al., 2001). Higher concentrations of 5-aza-dc should also be tested to see if both *Lhx3* gene transcripts could be expressed after 24 hours post treatment. Other GC-rich promoter regions should also be examined for occupation by both MeCP2 and other methyl-CpG binding proteins. It would be interesting to see if higher doses of 5-

aza-dc plus TSA eventually led to reactivation of the *Lhx3* gene and its downstream pituitary gene targets. Also, one could study if DNMT1 is the methyltransferase regulating the *Lhx3* gene expression in the Pit-1/0 cells by using DNMT1 inhibitors, such as DNMT1 small interfering RNA (siRNA).

Other studies could be designed to further explore the interactions between HDACs, MeCP2 and the *Lhx3* promoters. As mentioned earlier, MeCP2 binds AT-rich DNA sequences and the *Lhx3* enhancer is AT-rich. Therefore, a further study using Combined 3C-ChIP-Cloning (6C) technology could test whether or not MeCP2 modulates this interaction. Co-immunoprecipitation assays could be performed to confirm interaction of MeCP2 with HDACs in Pit-1/0 cells, and sequential chromatin immunoprecipitation assays (also known as re-chip assays) could be performed to determine if HDAC and MeCP2 proteins simultaneously bind the same region of the *Lhx3* promoter. Additionally, ChIP assays could also be performed to examine the occupation of the *Lhx3* promoters by other histone markers, such as methylation at lysine 9 of histone H3, as MeCP2 has also been shown to recruit histone methyltransferases to this site (Lunyak et al., 2002; Fuks et al., 2003). 5mc can be oxidized to form 5-hydroxymethyl-cytosine (5hmC), which is up regulated in neuronal tissues (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009; Nestor et al., 2012) and the conversion can be detected by chromatography coupled to mass spectroscopy or 5hmC DNA immunoprecipitation. Mellén and colleagues recently showed that MeCP2 binds 5hmC in neuronal tissues (Mellen et al., 2012). *Lhx3* mRNA has been detected in ventral spinal cord, the pons, the medulla oblongata and the pineal gland, so further analysis of the gene should be conducted to determine if it contains the 5hmC modification and if MeCP2

binds the 5hmC modified DNA in these tissues (Seidah et al., 1994; Zhadanov et al., 1995).

LHX3 plays a critical role in pituitary development, and mutations in human *LHX3* lead to combined pituitary hormone deficiency disease (CPHD). Therefore, discovering cellular processes that potentially influence *LHX3/Lhx3* expression, such as DNA methylation contribute to our understanding of pituitary organogenesis. Additionally, this study identifies potential candidate regions of the *Lhx3* gene that may be used in the diagnosis of CPHD patients of unknown etiology.

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